

## OLIGONUCLEOTIDE LABELING REACTANTS AND THEIR USE

### FIELD OF THE INVENTION

This invention relates to novel compounds and methods for labeling of oligonucleotides using machine assisted solid phase chemistry.

### 5 BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

10 Synthetic oligonucleotides tethered to various ligands have been used as research tools in molecular biology [see e.g.: Goodchild, *Bioconjugate Chem.*, **1990**, 3, 166; Uhlman and Peyman, *Chem. Rev.*, **1990**, 90, 543; Sigman, et al. *Chem. Rev.*, **1993**, 93, 2295; O'Donnel and McLaughlin in *Bioorganic Chemistry, Nucleic Acids*, Hecht SM, ed. Oxford Univ. Press, 1996, p. 216]. They have been applied to genetic analysis, and to elucidate mechanism of gene function. Oligonucleotides  
15 carrying reporter groups have had widespread use for automated DNA sequencing, hybridization affinity chromatography and fluorescence microscopy. Oligonucleotide-biotin conjugates are widely used as hybridization probes. Antisense oligonucleotides covalently linked to intercalators, chain cleaving or alkylating agents have been shown to be efficient as gene expression regulators. The  
20 sequence specific artificial nucleases, when targeted against mRNA, may find applications even as chemotherapeutics.

For several applications, such as in DNA hybridization assays, it is desirable to introduce more than one reporter group to the oligonucleotide structure. This can be performed by three alternative methods:

- 5 (i) by coupling several base- or carbohydrate-tethered nucleosidic building blocks to the growing oligonucleotide chain,
- (ii) by functionalization of the internucleosidic phosphodiester linkages, or
- (iii) by using several multifunctional non-nucleosidic building blocks during the oligonucleotide chain assembly.

10 All of these methods have their own drawbacks. Since the double helix formation of DNA is based on hydrogen bonding between the complementary base residues, tethers attached to the base moieties often weaken these interactions. This problem is easily overcome by using the tethered nucleosides at the 3'- or 5'-terminus of the coding sequence, or by using labels linked to C5 of pyrimidine residues. Introduction of tethers to the phosphate backbone gives rise to new chiral centers  
15 and makes the purification of these analogues difficult. Introduction of the tether arm to the carbohydrate moiety, in turn, often decreases the coupling efficiency of the phosphoramidite (steric hindrance). Furthermore, synthesis of these blocks is commonly extremely laborious. Although design of non-nucleosidic blocks may look attractive on paper, very often their syntheses suffer from complexity, low  
20 coupling yields and problems associated with the storage and handling of the phosphoramidites. For commercial applications design of base tethered nucleosidic building blocks is often the method of choice.

Introduction of linker arms to the nucleobase is most commonly performed by allowing a nucleoside with a good leaving group (*N*-tosyl, *N*-benzoyl, halogen,  
25 triazole, thiol) at C4 of pyrimidines or C2, C8 or C6 of purines to react with the appropriate nucleophilic linker molecule (*e.g.* an alkane- $\alpha,\omega$ -diamine). Since normally an excess of linker molecule and rather vigorous reaction conditions has to be used, laborious purification procedures cannot be avoided. The basic reaction conditions needed gives additional requirements to the protecting groups in the

target molecule. These problems may be overcome by attachment of the linker molecules to C5 of pyrimidine bases by a palladium catalyzed coupling reaction between 5-halogeno pyrimidine 5-mercuriochloro nucleoside and an alkynyl or allyl linker, respectively. However, the method involves rather laborious synthesis of a 5-halogeno or 5-mercuriochloro nucleoside. Very recently, attachment of a linker arm to the N3 of 3',5'-O-protected thymidine based on Mitsunobu reaction [Mitsunobu, *Synthesis*, **1981**, 1] was reported [*J. Org. Chem.*, **1999**, 64, 5083; *Nucleosides, Nucleotides*, **1999**, 18, 1339]. Since the coupling reaction is performed under mild conditions, a wide range of tether arms can be introduced.

- 10 Most of the methods for oligonucleotide tethering described in literature involves attachment of functional groups in the oligonucleotide structure during chain assembly. Hence, introduction of the label molecules has to be performed in solution. In the labeling reaction the additional amino or mercapto groups of oligonucleotides are allowed to react in solution with isothiocyanato, haloacetyl or 15 2,4,6-triazinyl derivatives of label molecules. Carboxylic acid groups, in turn, can be labeled with amino tethered labels with the aid of water-soluble carbodiimide. Since in all the cases the labeling reaction is performed in aqueous solution with an excess of labeling reactants, laborious purification procedures cannot be avoided. Especially when attachment of several labels is required the isolation and 20 characterization of the desired conjugate is extremely difficult, and often practically impossible. Hence, several attempts to incorporate label molecules or their appropriately protected precursor to oligonucleotide structure during chain assembly have been done [Ruth, JL et al, US 4,948,882; Brush, CK et al, US 5,583,236]. The fluorescent label monomers for solid phase chemistry synthesized 25 are most commonly organic dyes (e.g. fluorescein, rhodamine, dansyl, dabsyl, pyrene, TAMRA) several of these are even commercially available. However, such labels and labeled biomolecules suffer from many commonly known drawbacks such as Raman scattering, other fluorescent impurities, low water solubility, concentration quenching etc. In the specific binding assays, generally very low 30 concentrations of analytes to be measured are present. Thus multilabeling of

oligonucleotides with organic fluorophores may not enough enhance detection sensitivity needed in several applications. For these types of applications lanthanide(III) chelates are labels of choice since they do not suffer from this phenomenon. In DNA hybridization assays, time-resolved luminescence spectroscopy using lanthanide chelates is well known [Hemmilä et al. *Bioanalytical Applications of Labelling Technologies*, Wallac Oy, **1994**]. Therefore, a number of attempts have been made to develop non-luminescent (DELFI<sup>®</sup>) and new highly luminescent chelate labels suitable for time-resolved fluorometric applications. Many patent publications disclose non-luminescent labels [e.g. EP 0064484 A2, EP 0139675 B1, EP 0298939 A1, US 4,808,541 and US 4,565,790]. Highly luminescent labels include e.g. stable chelates composed of derivatives of pyridines [US 4,920,195, US 4,801,722, US 4,761,481, WO 93/11433, US 4,459,186, EP 0770610 A1 and Remuinan et al, *J. Chem. Soc. Perkin Trans 2*, **1993**, 1099], bipyridines [US 5,216,134], terpyridines [US 4,859,777, US 5,202,423 and US 5,324,825] or various phenolic compounds [US 4,670,572, US 4,794,191 and Ital. Pat. 42508 A789] as the energy mediating groups and polycarboxylic acids as chelating parts. In addition, various dicarboxylate derivatives [US 5,032,677, US 5,055,578 and US 4,772,563] macrocyclic cryptates [US 4,927,923, WO 93/5049 and EP 0493745 A1] and macrocyclic Schiff bases [EP 369000 A] have been patented. Also a method for labeling of biospecific binding reactants such as hapten, a peptide, a receptor ligand, a drug or PNA oligomer with luminescent labels by using solid-phase synthesis has been published [EP 067205A1]. One such oligonucleotide labeling reagent has been synthesized and used in multilabeling of oligonucleotides [Kwiatkowski et al. *Nucleic Acids Res.*, **22**, **1994**, 2604]. However the synthetic strategy described allows only preparation of chelates where the nucleobase is conjugated to the chelate structure limiting the chelate stability and versatility. Furthermore, the structure synthesized is usable only with europium(III) but not with terbium(III), dysprosium(III) or samarium(III).

For some special applications such as helicase assays based on fluorescence energy transfer [Earnshaw et. al, *J. Biomol. Screening*, **4**, **1999**, 239] large quantities of

ultrapure oligonucleotides bearing a luminescent lanthanide(III) chelate at their 3'- or 5'-terminus are needed. Although these molecules can be obtained by classical labeling methods in solution, yields of the oligonucleotide conjugates can be dramatically improved and purification procedures can be highly simplified if the label could be attached to the oligonucleotide structure during chain assembly. For 5'-derivatization synthesis of nucleosidic or non-nucleosidic building blocks are needed, while 3'-labeling calls for appropriately derivatized polymeric solid supports.

## OBJECTS AND SUMMARY OF THE INVENTION

10 The main objective of the present invention is to improve labeling of oligonucleotides with a desired number of lanthanide(III) chelates.

One objective of the invention is to provide improved labeling reactants for labeling an oligonucleotide.

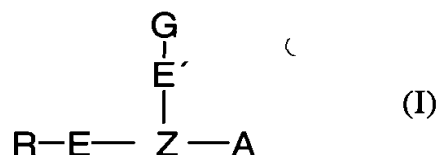
Another objective of the invention is to provide a highly simplified method for the preparation of nucleosidic building blocks that allow large-scale preparation of oligonucleotide conjugates containing additional functional groups in their structure.

The invention provides improved labeling reactants and a versatile method for direct attachment of a desired number of conjugate groups to the oligonucleotide structure during chain assembly. Hence solution phase labeling and laborious purification procedures can be avoided. The key reaction in the synthetic strategy towards nucleosidic oligonucleotide building blocks is a Mitsunobu alkylation which allows introduction of various labeling reactants to the nucleoside, and finally to the oligonucleotide structure. When oligonucleotides labeled with lanthanide(III) chelates are synthesized, initially precursors of lanthanide(III) chelates are introduced to the oligonucleotide structure during chain assembly, and

they are converted to the corresponding lanthanide(III) chelates during deprotection steps.

For some applications, e.g. for helicase assays, ultrapure oligonucleotides bearing a single label molecule at 3'- or 5'-terminus are needed. The present approach for the introduction of lanthanide(III) chelates at these positions on solid phase is also demonstrated.

Thus, the present invention concerns a labeling reactant of formula (I) suitable for labeling an oligonucleotide.



10 Wherein:

**R** is a temporary protecting group such as 4,4'-dimethoxytrityl (DMTr), 4-methoxytrityl (MMTr), trityl (Tr), (9-phenyl)xanthen-9-yl (pixyl) or not present.

**A** is either a phosphorylating moiety  $\text{---O---}\overset{\text{L}}{\underset{\text{L}''}{\underset{\text{L}'''}{\text{P}}}}\text{---}$  where

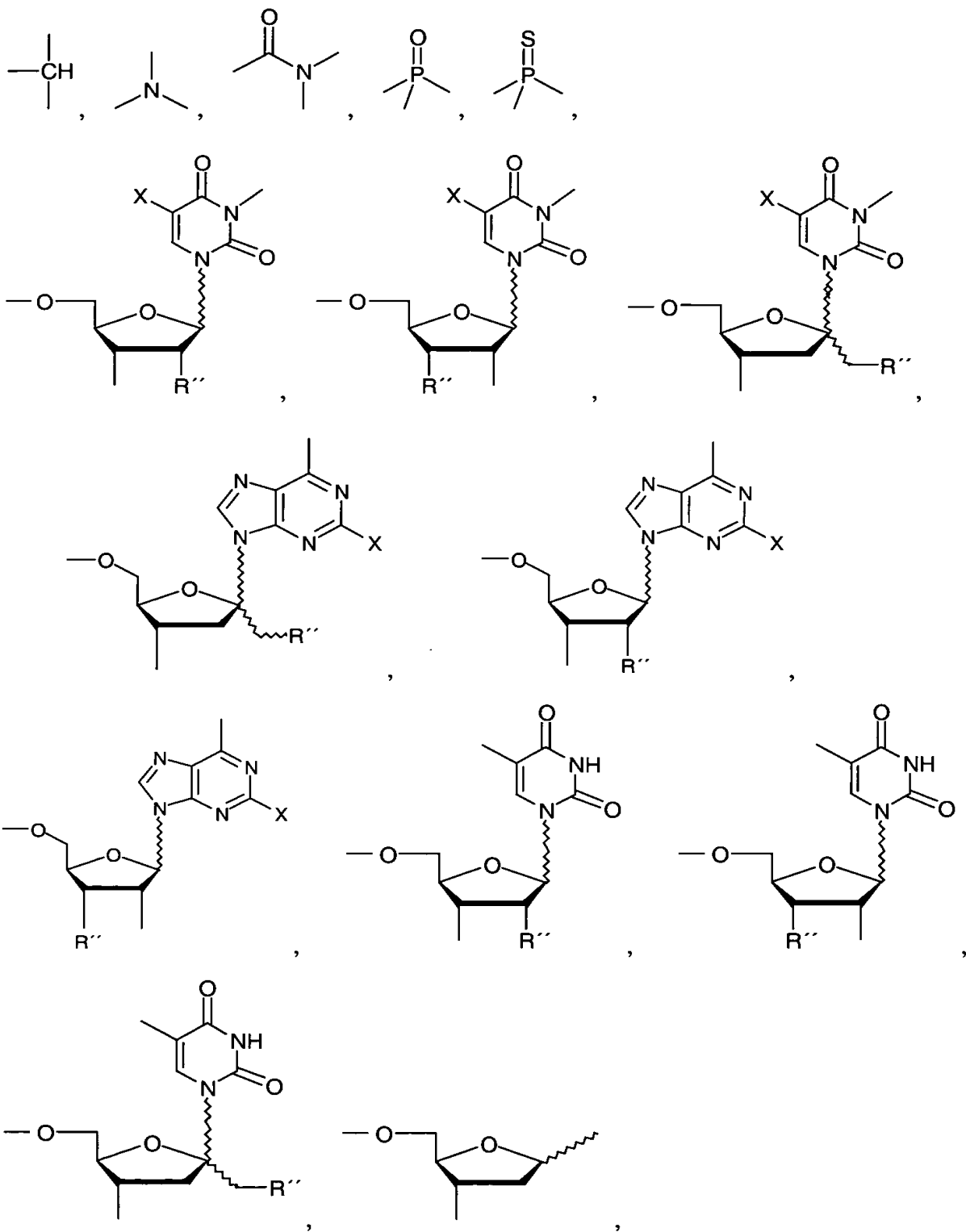
**L** is O, S, or not present

15 **L'** is H,  $\text{L}'''\text{CH}_2\text{CH}_2\text{CN}$  or  $\text{L}'''\text{Ar}$ , where Ar is phenyl or its substituted derivative, where the substituent is nitro or chlorine, and  $\text{L}'''$  is O or S;

**L''** is  $\text{O}^-$ ,  $\text{S}^-$ , Cl,  $\text{N}(i\text{-Pr})_2$ ; or

**A** is a solid support tethered to **Z** via a linker arm, which is formed of one to ten moieties, each moiety being selected from a group consisting of phenylene, alkylene containing 1–12 carbon atoms, ethynediyl ( $-\text{C}\equiv\text{C}-$ ), ether ( $-\text{O}-$ ), thioether ( $-\text{S}-$ ), amide ( $-\text{CO}-\text{NH}-$ ,  $-\text{NH}-\text{CO}-$ ,  $-\text{CO}-\text{NR}'-$  and  $-\text{NR}'-\text{CO}-$ ), carbonyl ( $-\text{CO}-$ ), ester ( $-\text{COO}-$  and  $-\text{OOC}-$ ), disulfide ( $-\text{S}-\text{S}-$ ), diaza ( $-\text{N}=\text{N}-$ ), and tertiary amine ( $-\text{N}-\text{R}'$ ), wherein  $\text{R}'$  represents an alkyl containing less than 5 carbon atoms.

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or trivalent derivatives, substituted or unsubstituted, of cyclohexane, cyclohexene, cyclohexadiene, phenyl, cyclopentane, cyclopentene, cyclopentadiene, cyclobutane, cyclobutene, cyclobutadiene, aziridine, diaziridine, oxetane, thietaneazete, azetidine, 1,2-dihydro-1,2-diazete, 1,2-diazetidene, furan, tetrahydrofuran, thiophene, 2,5-dihydrothiophene, thiolane, selenophene, pyrrole, pyrrolidine, phosphole, 1,3-dioxolane, 1,2-dithiole, 1,2-thiolane, 1,3-dithiole, 1,3-dithiolane, oxazole, 4,5-dihydrooxazole, isoxazole, 4,5-dihydroisoxazole, 2,3-dihydroisoxazole, thiazole, isothiazole, imidazole, imidazolidine, pyrazole, 4,5-dihydropyrazole, pyrazolidine, triazole, pyran, pyran-2-one, 3,4-dihydro-2H-pyran, tetrahydropyran, 4H-pyran, pyran-4-one, pyridine, pyridone, piperidine, phosphabenzene, 1,4-dioxin, 1,4-dithiin, 1,4-oxathiin, oxazine, 1,3-oxazinone, morpholine, 1,3-dioxane, 1,3-dithiane, pyridazine, pyrimidine, pyrazine, piperazine, 1,2,4-triazine, 1,3,5-triazine, 1,3,5-triaza-cyclohexane-2,4,6-trione; where

$R''$  is H or  $X'X''$ , where

$X'$  is -O-, -S-, -N-, ON- or -NH- and  $X''$  is a permanent protection group such as *t*-butyldimethylsilyl-, tetrahydropyranyl, 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl-, 1-[2-chloro-4-methylphenyl]-4-methoxypiperidin-4-yl-, 4-methoxytetrahydropyran-4-yl-, phthaloyl-, acetyl-, pivaloyl-, benzoyl-, 4-methylbenzoyl, benzyl-, trityl or

$X'$  is -O- and  $X''$  is alkyl or alkoxyalkylalkyl;

$X$  is H, alkyl, alkynyl, allyl, Cl, Br, I, F, S, O,  $NHCOCH(CH_3)_2$ ,  $NHCOCH_3$ ,  $NHCOPh$ ,  $SPh_3$ ,  $OCOCH_3$  or  $OCOPh$ .

$E$  is a linker arm between  $R$  and  $Z$ , and is formed of one to ten moieties, each moiety being selected from a group consisting of phenylene, alkylene containing 1–12 carbon atoms, ethynediyl ( $-C\equiv C-$ ), ether ( $-O-$ ), thioether ( $-S-$ ), amide ( $-CO-NH-$ ,  $-NH-CO-$ ,  $-CO-NR'-$  and  $-NR'-CO-$ ), carbonyl ( $-CO-$ ), ester ( $-COO-$  and  $-OOC-$ ), disulfide ( $-S-S-$ ), diaza ( $-N=N-$ ), and tertiary amine ( $-N-R'$ ), wherein  $R'$  represents an alkyl containing less than 5 carbon atoms, or not present.



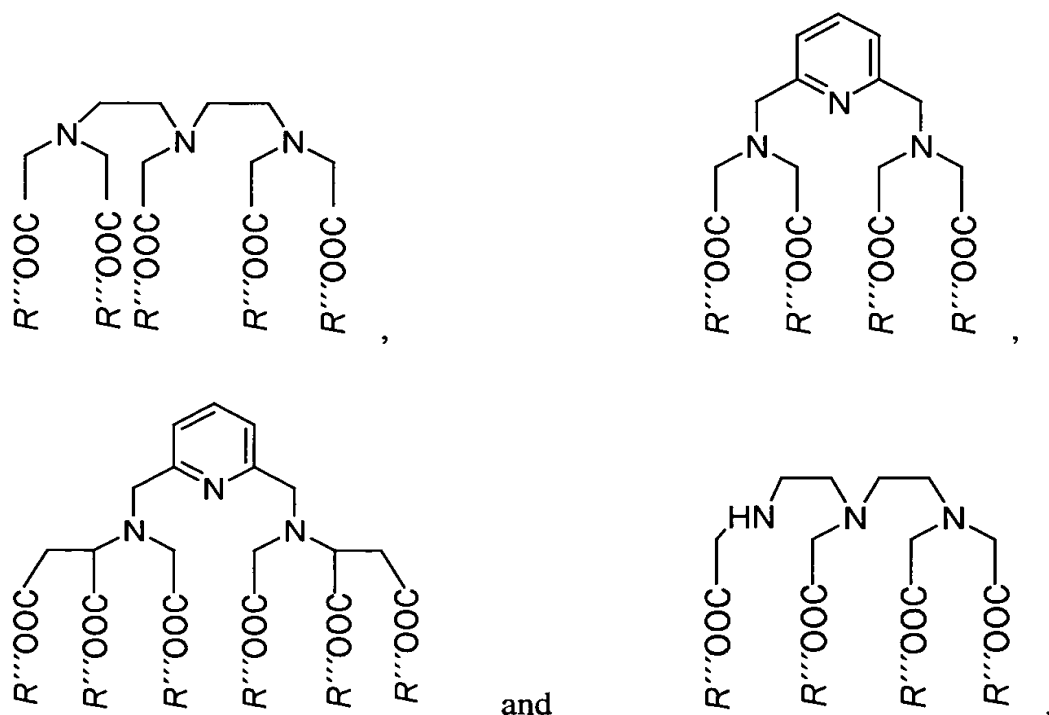
- $E'$  is a linker arm between  $G$  and  $Z$ , and is formed of one to ten moieties, each moiety being selected from a group consisting of phenylene, alkylene containing 1–12 carbon atoms, ethynediyl ( $-C\equiv C-$ ), ether ( $-O-$ ), thioether ( $-S-$ ), amide ( $-CO-NH-$ ,  $-NH-CO-$ ,  $-CO-NR'-$  and  $-NR'-CO-$ ), carbonyl ( $-CO-$ ), ester ( $-COO-$  and  $-OOC-$ ),  
 5 disulfide ( $-S-S-$ ), diaza ( $-N=N-$ ), and tertiary amine ( $-N-R'$ ), wherein  $R'$  represents an alkyl containing less than 5 carbon atoms, or not present.

$G$  is a bivalent aromatic structure, tethered to two iminodiacetic acid ester groups  $N(COOR'')_2$ , where

- 10  $R''$  is an alkyl of 1 to 4 carbon atoms, allyl, ethyltrimethylsilyl, phenyl or benzyl, which phenyl or benzyl can be substituted or unsubstituted, and

said bivalent aromatic structure is capable of absorbing light or energy and transferring the excitation energy to a lanthanide ion after the solid phase synthesis made labeling reactant has been released from the used solid support, deprotected and converted to a lanthanide chelate, or

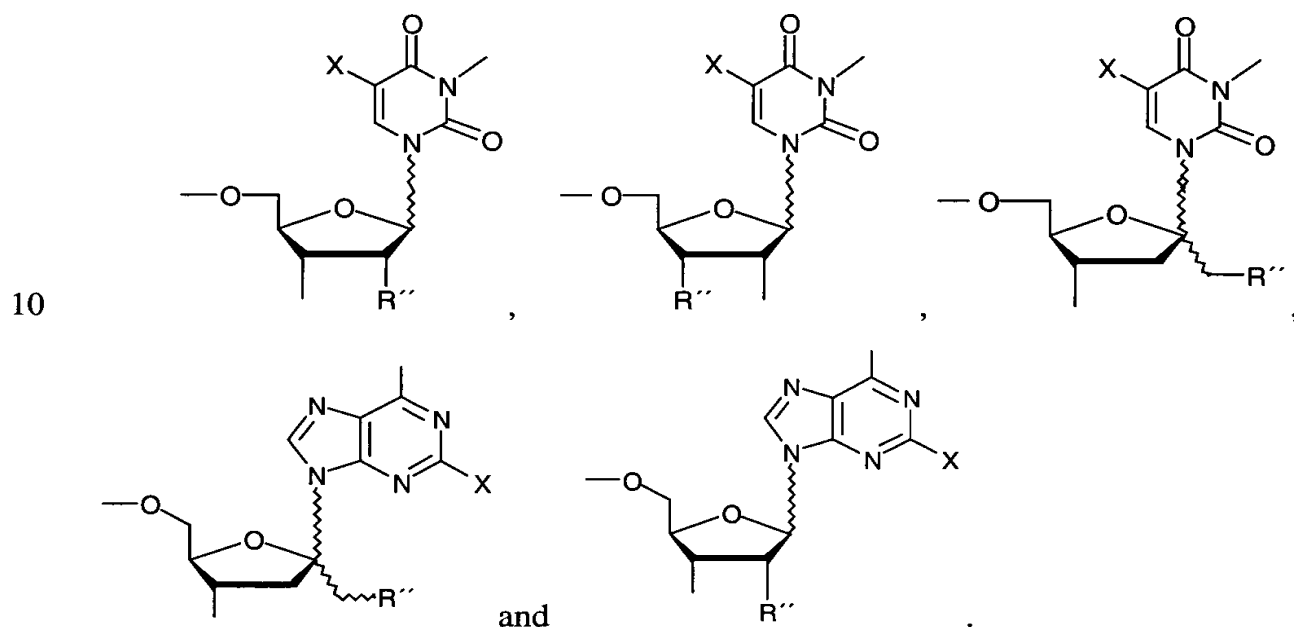
- 15  $G$  is a structure selected from a group consisting of



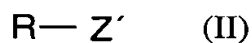
where

$R'''$  is an alkyl of 1 to 4 carbon atoms, allyl, ethyltrimethylsilyl, phenyl or benzyl, which phenyl or benzyl can be substituted or unsubstituted, and one of the hydrogen atoms is substituted with  $E'$ , or

- 5 **G** is a protected functional group, where the functional group is amino, aminooxy, carboxyl, thiol, and the protecting group is phthaloyl, trityl, 2-(4-nitrophenylsulfonyl)ethoxycarbonyl, fluorenylmethyloxycarbonyl, benzyloxycarbonyl or *t*-butoxycarbonyl for amino and aminooxy, alkyl for carbonyl and alkyl or trityl for thiol provided that bridge point **Z** is selected from a group consisting of



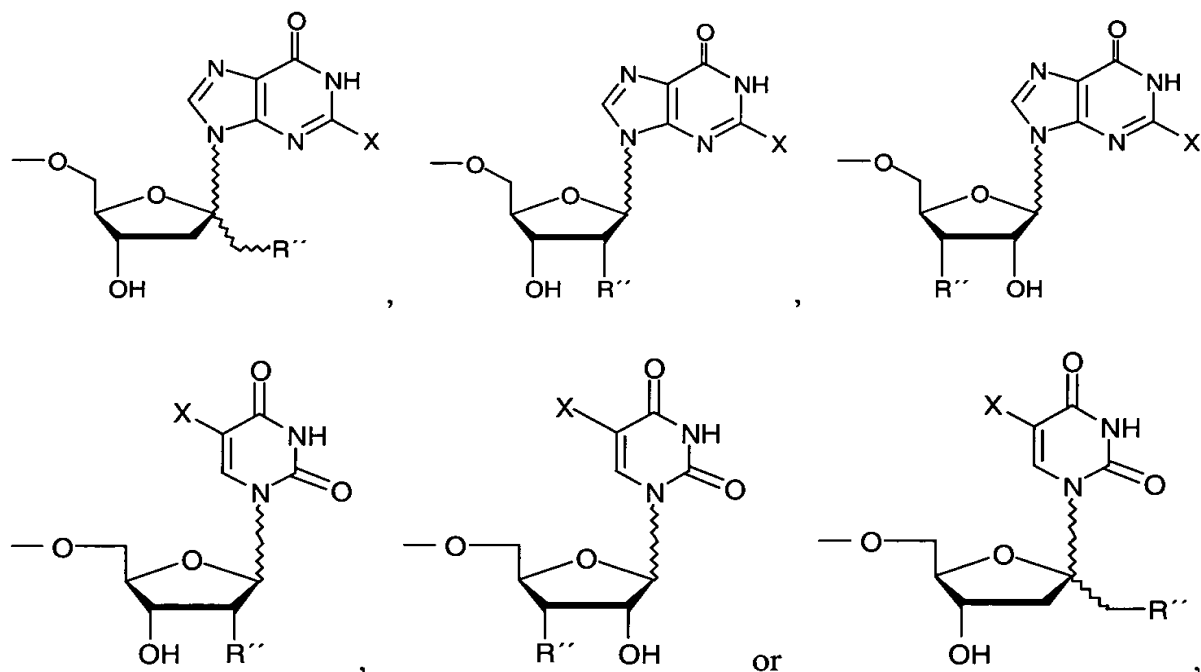
The present invention further concerns a method for direct attachment of a conjugate group to an oligonucleotide structure enabling the attachment of a desired number of these groups during chain assembly. Said method comprises a Mitsunobu  
15 alkylation of a compound of formula (II).



Wherein:

**R** is a temporary protecting group such as DMTr, MMTr, Tr, or pixyl.

**Z'** is an acidic bridge point selected from a group consisting of



5 where

**R''** is H or  $X'X''$ , where  $X'$  is -O-, -S-, -N-, ON- or -NH- and  $X''$  is a permanent protection group such as *t*-butyldimethylsilyl-, tetrahydropyranyl-, 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl-, 1-[2-chloro-4-methylphenyl]-4-methoxypiperidin-4-yl-, 4-methoxytetrahydropyran-4-yl-, phthaloyl-, acetyl-, pivaloyl-, benzoyl-, 4-methylbenzoyl, benzyl-, trityl or alkyl;

**X** is H, alkyl, alkynyl, allyl, Cl, Br, I, F, S, O,  $\text{NHCOCH}(\text{CH}_3)_2$ ,  $\text{NHCOCH}_3$ ,  $\text{NHCOPh}$ ,  $\text{SPh}_3$ ,  $\text{OCOCH}_3$  or  $\text{OCOPh}$ ;

and  $\text{pK}_a$  of said acidic bridge point is  $<14$ .

Said compound of formula (II) is alkylated with a compound of formula(III).

15  $\text{G}-\text{E}''$  (III)

Wherein:

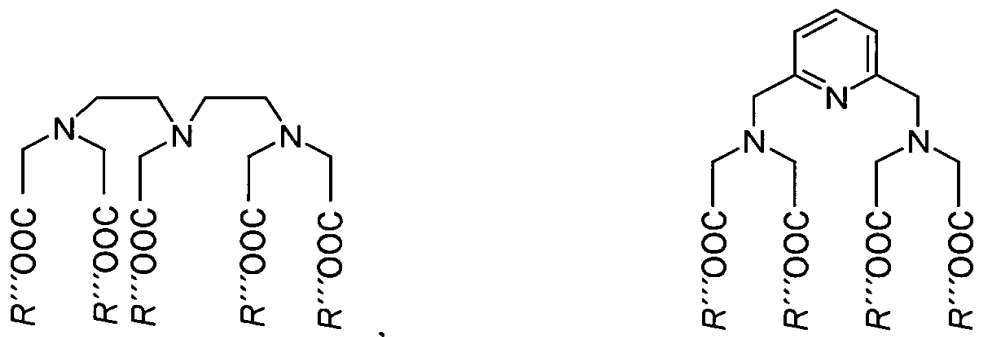
$E''$  is an arm with a primary aliphatic OH group at the end, which arm is formed of one to ten moieties, each moiety being selected from a group consisting of phenylene, alkylene containing 1–12 carbon atoms, ethynediyl ( $-C\equiv C-$ ), ether ( $-O-$ ), thioether ( $-S-$ ), amide ( $-CO-NH-$ ,  $-NH-CO-$ ,  $-CO-NR'-$  and  $-NR'-CO-$ ), carbonyl ( $-CO-$ ), ester ( $-COO-$  and  $-OOC-$ ), disulfide ( $-S-S-$ ), diaza ( $-N=N-$ ), and tertiary amine ( $-N-R'$ ), wherein  $R'$  represents an alkyl containing less than 5 carbon atoms.

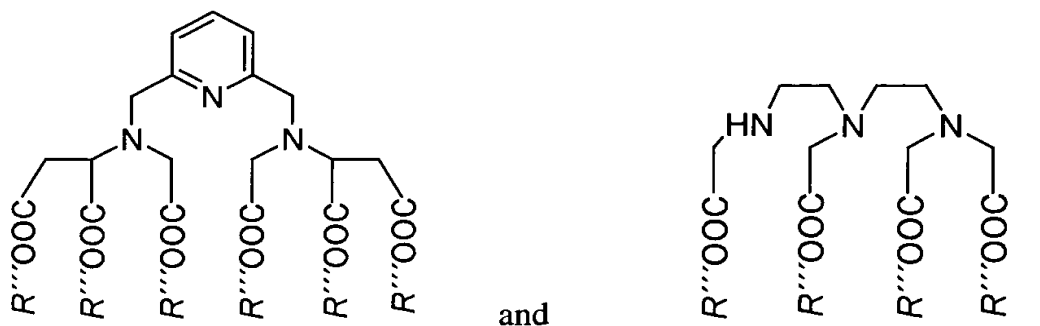
$G$  is a bivalent aromatic structure, tethered to two iminodiacetic acid ester groups  $N(COOR'')$ , where

$R''$  is an alkyl of 1 to 4 carbon atoms, allyl, ethyltrimethylsilyl, phenyl or benzyl, which phenyl or benzyl can be substituted or unsubstituted and

said bivalent aromatic structure is capable of absorbing light or energy and transferring the excitation energy to a lanthanide ion after the solid phase synthesis made labeling reactant has been released from the used solid support, deprotected and converted to a lanthanide chelate, or

$G$  is a structure selected from a group consisting of





where

$R'''$  is an alkyl of 1 to 4 carbon atoms, allyl, ethyltrimethylsilyl, phenyl or benzyl, which phenyl or benzyl can be substituted or unsubstituted, and

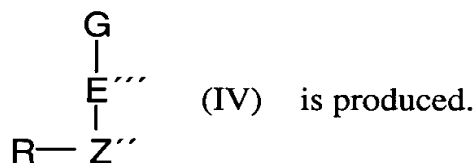
one of the hydrogen atoms is substituted with  $E'$ , or

**G** is a protected functional group, where the functional group is amino, aminooxy, carboxyl, thiol, and the protecting group is phthaloyl, trityl, 2-(4-nitrophenylsulfonyl)ethoxycarbonyl, fluorenylmethyloxycarbonyl, benzyloxycarbonyl or *t*-butoxycarbonyl for amino and aminooxy, alkyl for carbonyl and alkyl or trityl for thiol, or

**G** is not present.

The functional groups of  $E'$  and **G**, excluding said primary aliphatic OH group, are protected.

A compound of formula (IV)



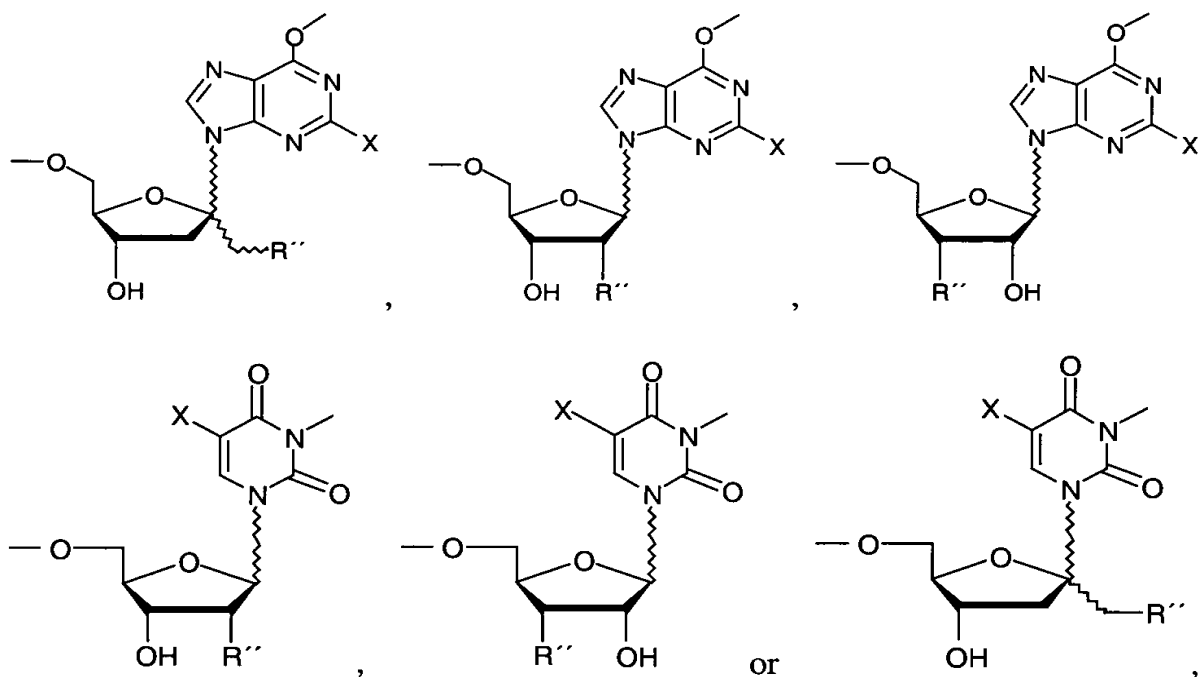
Wherein: **G** and **R** of compound (IV) are as defined above;

$E'''$  is a linker arm between **G** and **Z**, and is formed of one to ten moieties, each moiety being selected from a group consisting of phenylene, alkylene containing 1–

12 carbon atoms, ethynediyl ( $-\text{C}\equiv\text{C}-$ ), ether ( $-\text{O}-$ ), thioether ( $-\text{S}-$ ), amide ( $-\text{CO}-\text{NH}-$ ,  $-\text{NH}-\text{CO}-$ ,  $-\text{CO}-\text{NR}'-$  and  $-\text{NR}'-\text{CO}-$ ), carbonyl ( $-\text{CO}-$ ), ester ( $-\text{COO}-$  and  $-\text{OOC}-$ ), disulfide ( $-\text{S}-\text{S}-$ ), diaza ( $-\text{N}=\text{N}-$ ), and tertiary amine ( $-\text{N}-\text{R}'$ ), wherein  $\text{R}'$  represents an alkyl containing less than 5 carbon atoms; and

$\text{Z}''$  is a bridge point selected from a group consisting of

5



where

$\text{R}''$  is H or  $\text{X}'\text{X}''$ , where  $\text{X}'$  is  $-\text{O}-$ ,  $-\text{S}-$ ,  $-\text{N}-$ ,  $\text{ON}-$  or  $-\text{NH}-$  and  $\text{X}''$  is a permanent protection group such as *t*-butyldimethylsilyl-, tetrahydropyranyl, 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl-, 1-[2-chloro-4-methylphenyl]-4-methoxypiperidin-4-yl-, 4-methoxytetrahydropyran-4-yl-, phthaloyl-, acetyl, pivaloyl-, benzoyl-, 4-methylbenzoyl, benzyl-, trityl or alkyl;

$\text{X}$  is H, alkyl, alkynyl, allyl, Cl, Br, I, F, S, O,  $\text{NHCOCH}(\text{CH}_3)_2$ ,  $\text{NHCOCH}_3$ ,  $\text{NHCOPh}$ ,  $\text{SPh}_3$ ,  $\text{OCOCH}_3$  or  $\text{OCOPh}$ .

15

## ADVANTAGES AND KEY STEPS OF METHOD FOR OLIGONUCLEOTIDE DERIVATIZATION

The present invention for oligonucleotide derivatization combines several important features:

- 5 (i) The nucleosidic protected functional group tethered building blocks can be synthesized in a few days using cheap reagents, equimolar reagent ratios, and simple purification procedures. The starting materials are commercially available and can also be prepared in a single step using standard well-  
10 documented textbook protocols [Gait, M. Oligonucleotide Synthesis, a Practical Approach, IRL Press, 1990]. The key reaction in the present invention is the Mitsunobu alkylation of the above mentioned 5'-*O*-protected nucleoside and the appropriate linker molecule i.e. a primary alcohol where additional functional groups are protected. Under the reaction conditions employed 3'-*O*-protection of the nucleoside is not required. These  
15 nucleosides are finally converted to the corresponding phosphoramidites in conventional manner, and they can be purified either by precipitation from cold hexanes, or by silica gel column chromatography. Since the products are solids, their storage and handling does not suffer from the problems associated with oily non-nucleosidic phosphoramidites.
- 20 (ii) Since the coupling reaction between the nucleoside and the tether molecule is performed under mild reaction conditions [at ambient temperature in dry tetrahydrofuran (THF)] using equimolar reagent ratios, a wide range of tethers can be introduced. The only requirement is that the tether molecule has a primary hydroxyl group in its structure, and other functional groups are  
25 protected. Hence very complicated molecules can be incorporated to the nucleoside (and finally to the oligonucleotide structure) in high efficiency. These tethers with conjugate groups for different applications can be:
  - (a) fluorescent or chemiluminescent groups or spin-labels,

(b) chemically reactive groups that induce irreversible reactions to their target sequences, or

(c) groups that promote intermolecular interactions (*e.g.* biotin).

Representative structures synthesized according to the method of the present invention are presented in schemes 2–11.

(iii) Since the building blocks are derivatives of nucleosides bearing tether arm attached to the base moiety, they can be coupled to the oligonucleotide chain using standard protocols in high efficiency (*i.e.* no changes in concentrations or coupling times required).

(iv) Since the tether arm is attached to the base moiety, multilabeling of oligonucleotides is achievable.

(v) If a ligand structure/structures is/are incorporated to the oligonucleotide chain during chain assembly, it/they can be converted to the corresponding lanthanide(III) chelates during slightly modified deprotection steps. Hence laborious solution phase labeling as well as synthesis of the activated chelates and oligonucleotides tethered to functional groups can be avoided.

(vi) For several applications introduction of only a single label molecule at the 5'-terminus of the oligonucleotide structure is needed. For these applications the ligand structures can be simplified by omitting the nucleobase from the structure *i.e.* resulting in non-nucleosidic phosphoramidite building blocks. Examples of such a molecules are shown in examples 22 and 33.

(vii) For the preparation of 3'-tethered oligonucleotides the ligand structures can be converted also to the corresponding non-nucleosidic or nucleosidic solid supports that can be used in solid phase oligonucleotide synthesis. The solid support can be either a long chain alkylamine controlled pore glass (LCAA) or polystyrene. An example of such a solid support is shown in example 24.



(viii) Several of the structures described above can be obtained also by using slightly modified reaction routes:

- 5 (a) A nucleoside tethered to an alkynyl group is synthesized by Mitsunobu alkylation, the ligand structure is coupled to it as an aromatic halide using Sonagoshira reaction.
- (b) A nucleoside tethered to a protected functional group is synthesized using Mitsunobu reaction, the protecting group is selectively removed (e.g. ammonolysis for trifluoroacetyl-amido), and the ligand or label structure is coupled by carbodiimide assisted reaction.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The novel labeling reactants and labeling methods of the present invention are particularly suitable for the preparation of oligonucleotide conjugates bearing a desired known number of functional groups or label molecules in their structure.

15 The term 'bivalent' in the definition of **G** shall mean a chemical group bound to two neighboring atoms.

The functional groups most suitable are amino, carboxyl, aminooxy or thiol.

The most suitable chelates are non-luminescent and luminescent lanthanide(III) chelates.

20 The organic dyes suitable for monolabeling are dabsyl, dansyl, fluorescein, rhodamine or TAMRA.

A particularly preferable transient protecting group **R** is 4,4'-dimethoxytrityl

The sugar of the nucleotide is preferably ribose or 2-deoxyribose. In the former case the permanent protecting group **X''** for hydroxyl is preferably *t*-butyldimethylsilyl,

tetrahydropyranyl, 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl- (Fpmp), 1-[2-chloro-4-methylphenyl]-4-methoxypiperidin-4-yl- or 4-methoxytetrahydropyran-4-yl-, or X'' is an alkyl or alkoxalkyl, preferably methyl, methoxymethyl or etoxymethyl.

For luminescent labeling reactants **G** is a bivalent aromatic structure and is preferably selected from a group consisting of carbostyryl or structures disclosed in Scheme 1A. For non-luminescent labeling reactants **G** is selected from a group of structures disclosed in 1B.

The substituent R''' is preferably methyl, ethyl or allyl.

Most preferably, the labeling reactant is

10 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-N3 {tetramethyl 2,2',2'',2'''-[(4-(1-hexyn-5-yl)pyridine-2,6-diyl)bis(methylenenitrilo)]tetrakis(acetato) uridine 3'-O-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (7),

N3-[6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hex-1-yl-5'-O-(4,4'-dimethoxytrityl)thymidine 3'-O-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (12),

15 5'-O-(4,4'-dimethoxytrityl)-N3-{tetramethyl-2,2',2'',2'''-{6,6'-[4'-hydroxyethoxyethoxyphenylethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)tetrakis(acetato)}thymidine 3'-O-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (18),

20 tetramethyl-2,2',2'',2'''-{6,6'-[4-(6-hydroxyhexyl)-1H-pyrazol-1,3-diyl]bis(pyridine)-2,2'-diyl}bis(methylenenitrilo)}tetrakis(acetato)-6-O-(2-cyanoethyl) N,N-diisopropylphosphoramidite (25),

2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-3,6-{4-{6,6''-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]-2,2':6',2''-terpyridine-4'-yl}phenyl}hex-5-yn-1-yl}-uridine 3'-[O-(2-cyanoethyl)-N,N-diisopropyl]phosphoramidite (37) or

25 6-{4-{6,6''-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]-2,2':6',2''-terpyridine-4'-yl}phenyl}hex-5-yn-1-ol [O-(2-cyanoethyl)-N,N-diisopropyl]-phosphoramidite (38).

Most preferably the solid support is 5'-O-(4,4'-dimethoxytrityl)-3'-O-succinyl-N3-{tetramethyl-2,2',2'',2'''-{6,6'-[4'-hydroxyethoxyethoxyphenylethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)tetrakis(acetato)}thymidine long chain alkylamine controlled pore glass (24).

- 5 According to a preferred embodiment the lanthanide chelate is a europium(III), terbium(III), samarium(III) or dysprosium(III) chelate.

The invention is further elucidated by the following examples. The structures and synthetic routes employed in the experimental part are depicted in schemes 2–9. Scheme 2 illustrates the synthesis of the labeling reagents 3 and 4. The experimental details are given in examples 1–4. Schemes 3A and 3B illustrate the synthesis of the labeling reagent 8. Scheme 4 illustrates synthesis of the labeling reagent 12. The experimental details are given in examples 10–12. Schemes 5A and 5B illustrate the preparation of the labeling reagent 18. Experimental details are given in examples 13–16. Scheme 6A and 6B illustrate the synthesis of labeling reagent 25. Experimental details are given in examples 18–22. Scheme 7A and 7B illustrate the synthesis of the solid support 27. Experimental details are presented in examples 23 and 24. Scheme 8A and 8B illustrate the synthesis of labeling reagents 37 and 38. Experimental details are given in examples 25–33. Scheme 9 illustrates the introduction of primary amino groups to the oligonucleotide structure in the aid of compound 8 as well as further oligonucleotide derivatization in solution. Experimental details are given in example 35. Scheme 10 illustrates introduction of lanthanide(III) chelates to the oligonucleotide structure in with the aid of compound 8. Experimental details are given in example 36. Scheme 11 illustrates introduction of lanthanide(III) chelates to the oligonucleotide structure in with the aid of compound 38. Experimental details are given in example 37.

## EXPERIMENTAL PROCEDURES

Reagents for machine assisted oligonucleotide synthesis were purchased from PE Biosystems (Foster City, CA). 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite, *N*6-trifluoroacetamidohexanol and 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-uridine and 5'-*O*-(4,4'-dimethoxy-trityl)thymidine were synthesized according to published procedures. Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). NMR spectra were recorded on a Jeol LA-400 spectrometer operating at 399.8, 350, 161.9 and 100.5 MHz for  $^1\text{H}$ ,  $^{19}\text{F}$ ,  $^{31}\text{P}$  and  $^{13}\text{C}$ , respectively, or on a Jeol GX 500 instrument operating at 500.00 and 125.65 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively.  $\text{Me}_4\text{Si}$  was used as an internal ( $^1\text{H}$  and  $^{13}\text{C}$ ) and  $\text{H}_3\text{PO}_4$  ( $^{31}\text{P}$ ) and trifluoroacetic acid ( $^{19}\text{F}$ ) as external references. Coupling constants are given in Hz. When reported, signal characterization is based on  $^1\text{H}$ ,  $^1\text{H}$ ,  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}$ ,  $^{13}\text{C}$  COSY experiments. IR spectra were recorded on a Perkin Elmer 2000 FT-IR spectrophotometer. Fast atom bombardment mass spectra were recorded on a VG ZabSpec-ao TOF instrument in the positive detection mode. Oligonucleotides were assembled on an Applied Biosystems 932 DNA Synthesizer using phosphoramidite chemistry and recommended protocols (DMTr-Off-synthesis).

### Example 1

The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3-(*N*6-trifluoroacetamidohex-1-yl)uridine (1)

2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)uridine (8.0 g, 15.1 mmol),  $\text{Ph}_3\text{P}$  (4.7 g, 17.9 mmol) and *N*6-trifluoroacetamidohexan-1-ol (4.1 g, 18.1 mmol) were dissolved in dry THF (80 ml). DEAD (2.85 ml) was added in five portions during 15 min, after which the mixture was stirred 2 h at ambient temperature and concentrated. Purification on silica gel (eluent diethyl ether) yielded 65 % of 2.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ; 500 MHz):  $\delta$  9.41 (1H, br, NH); 7.72 (1H, d, H-6); 7.35 (2H,

DMTr); 7.25 (7H, DMTr); 6.85 (4H, d, DMTr); 5.5 (1H, d, H-5), 6.2 (1H, t, H-1'), 5.4 (1H, d, 3'-OH), 4.3 (1H, m, H-4'), 4.3 (1H, m, H-3'), 3.9 (1H, m, H-4'), 3.5 (1H, dd, H-5'), 3.8 (2H, t), 3.2 (1H, H-5'), 3.15 (2H, m), 2.2 (2H, H-2'; H-2'), 1.5 (4H, m); 1.25 (4H, m). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>): δ 161.7 (C4), 158.0 (C=O), 156.5 (q, CF<sub>3</sub>); 150.3 (C2); 144.8 (DMT); 138.8 (C6); 129.7, 127.8, 127.7, 126.7, 113.1 (DMT); 100.7 (C-5), 85.7 (DMT); 85.5 (C4'); 85.2 (C1'); 69.8 (C3'); 63.3 (C5'); 55.5 (2 · OMe); 40.1 (NCH<sub>2</sub>); 39.7 (C2'); 39.0 (CH<sub>2</sub>NHCO); 28.0, 25.9, 25.8 (CH<sub>2</sub>) <sup>15</sup>N NMR (DMSO-d<sub>6</sub>): δ-294.5 (NHCOCF<sub>3</sub>); -264.0 (N1); -244.6 (N3).

### Example 2

10 The synthesis of 5'-O-(4,4'-dimethoxytrityl)-N3-(N6-trifluoroacetamido)hexyl-thymidine (2)

The title compound was synthesized as described in example 1 for compound 1 by using 5'-O-(4,4'-dimethoxytrityl)thymidine as the starting material. The yield was 76 %. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>; 500 MHz): δ 9.35 (1H, br t, *J* 5.2, NH); 7.54 (1H, d, *J* 1.1, H-6); 7.38-7.23 (9H, DMTr); 6.88 (4H, d, DMTr); 6.22 (1H, t, *J* 6.6, H-1'); 5.31 (1H, d, *J* 4.6, 3'-OH); 4.31 (1H, m, H-3'); 3.89 (1H, m, H-4'); 3.77 (2H, m, NCH<sub>2</sub>); 3.72 (6H, s, 2 · OCH<sub>3</sub>); 3.21 (1H, dd, *J* 5.8 and 10.6 H-5'); 3.16 (1H, dd, *J* 3.0 and 10.6, H-5'); 3.15 (2H, m, CH<sub>2</sub>NH); 2.24 (1H, m, H-2'); 2.17 (1H, m, H-2'); 1.49 (3H, d, *J* 1.1 5-CH<sub>3</sub>); 1.48 (2H, m, NCH<sub>2</sub>CH<sub>2</sub>); 1.45 (2H, m, CH<sub>2</sub>CH<sub>2</sub>NH); 1.26 (4H, m, 2 · CH<sub>2</sub>). <sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ: 162.5 (C4), 158.1 (C=O), 156.1 (q, *J*<sub>C,F</sub> 35.9, CF<sub>3</sub>); 150.2 (C2); 144.7 (DMT); 134.3 (C6); 129.7, 127.8, 127.66, 126.7, 113.1 (DMT); 108.7 (C-5), 85.7 (DMT); 85.6 (C4'); 84.8 (C1'); 70.4 (C3'); 63.7 (C5'); 55.0 (2 · OMe); 40.4 (NCH<sub>2</sub>); 39.7 (C2'); 39.0 (CH<sub>2</sub>NHCO); 28.0, 26.9, 25.9, 25.8 (CH<sub>2</sub>).

**Example 3**

The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3-(*N*6-trifluoroacetamido-hexyl)uridine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite (**3**)

Predried compound **1** and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphordiamidite  
 5 (1.5 eq) were dissolved in dry acetonitrile. 1*H* tetrazole (1eq; 0.45 M in acetonitrile) was added, and the mixture was stirred for 30 min at room temperature before being poured into 5 % NaHCO<sub>3</sub> and extracted with dichloromethane and dried over Na<sub>2</sub>SO<sub>4</sub>. Precipitation from cold (−70 °C) hexane yielded the title compound as a white powder. Compound **3**: <sup>31</sup>P NMR (CDCl<sub>3</sub>): δ 148.6 (0.5 P), 148.4 (0.5 P).

**Example 4**

The synthesis of 5'-*O*-(4,4'-dimethoxytrityl)-*N*3-(*N*6-trifluoroacetamido)hexyl-thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl)phosphoramidite (**4**)

Phosphitylation of compound **2** as described in example 3 for compound **1** yielded the title compound as a white powder. Compound **4**: <sup>31</sup>P NMR (CDCl<sub>3</sub>): δ 148.6  
 15 (0.5 P), 148.4 (0.5 P).

**Example 5**

The synthesis of tetramethyl 2,2',2'', 2'''-[4-(6-hydroxyhex-5-yn-1-yl)pyridine-2,6-diyl]bis(methylenenitrilo)]tetrakis(acetate) (**6**)

A mixture of tetramethyl 2,2',2'',2'''-[4-bromopyridine-2,6-diyl]bis(methylene-  
 20 nitrilo)tetrakis(acetate) (**5**), bis(triphenylphosphinepalladium(II) chloride and CuI in dry THF and triethylamine was deaerated with argon. 5-hexynol was added and the mixture was stirred for 7 h at 55 °C. The cooled solution was filtered; the filtrate was evaporated and redissolved in dichloromethane. The solution was washed with water, dried and concentrated. Purification on silica gel yielded the title compound

as an oil (75 %). Compound **6**:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ; 400 MHz): 7.46 (2H, s); 3.99 (4H, s); 3.71 (12H, s, 4  $\text{CH}_3$ ); 3.62 (8H, s, 4  $\text{CH}_2$ ); 2.53 (4H, m,  $\text{CH}_2$ ); 1.70 (4H, m, 2  $\text{CH}_2$ ) IR (neat): 2242 ( $\text{C}\equiv\text{C}$ ).

### Example 6

- 5 The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3 {tetramethyl 2,2',2'', 2'''-[(4-(hex-5-yn-1-yl)pyridine-2,6-diyl)bis(methylenenitrilo)]tetrakis(acetato)-uridine (**7**) — Method A

2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)uridine was allowed to react with compound **6** as described in example 1. Purification on silica gel (eluent  $\text{CH}_2\text{Cl}_2$ : MeOH  
10 95:5, v/v) yielded the title compound as foam. The yield was 70 %. Compound **7**:  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ; 500 MHz):  $\delta$  7.67 (1H, d,  $J$  8.2, H-6); 7.36 (2H, s, pyridine); 7.35 (2H, DMTr); 7.25 (7H, DMTr); 6.85 (4H, d, DMTr); 6.16 (1H, t, H-1,  $J$  6.3); 5.48 (1H, d,  $J$  8.1, H-5); 5.34 (1H, d,  $J$  4.8, 3'-OH); 4.29 (1H, m, H-3'); 3.89 (1H, m, H-4'); 3.86 (4H, s, 2  $\cdot$   $\text{CH}_2$ ); 3.82 (2H, t,  $J$  5.6 Ar- $\text{CH}_2$ ); 3.58 (8H, s, 4  $\cdot$   $\text{CH}_2$ ),  
15 3.24 (1H, dd,  $J$  10.7 and 5.2, H-5'); 3.19 (1H, dd,  $J$  3.1 and 10.7, H-5''); 2.50 (2H, t,  $\text{CH}_2$ ); 2.20 (2H, t, H-2' and H-2''); 2.72 (1H, br, OH) 1.73 (2H, m,  $\text{CH}_2$ ); 1.52 (2H, m,  $\text{CH}_2$ ).

### Example 7

Synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3 (hex-5-yn-1-yl) uridine (**9**)

- 20 5-Hexynol was allowed to react with 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)uridine under Mitsunobu conditions described in example 1. Purification on silica gel (eluent diethyl ether) yielded the title compound as a solid (86 %) Compound **9**:  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 400 MHz):  $\delta$  7.70 (1H, d,  $J$  8.1, H-6); 7.39 (2H, DMT); 7.31 (2H, DMT); 7.25 (5H, DMT); 6.90 (4H, d,  $J$  8.0); 6.18 (1H, t,  $J$  6.3, H-1');  
25 5.49 (1H,  $J$  8.1, H-5); 5.38 (1H, d,  $J$  4.6, 3'-OH); 4.31 (1H, m, H-3'); 3.90 (1H, m,

H-4'); 3.78 (2H, m, NCH<sub>2</sub>); 3.74 (6H, s, 2 · OCH<sub>3</sub>); 3.26 (1H, dd, *J* 5.4 and 10.7, H-5'); 3.19 (1H, dd, *J* 2.9 and 10.7, H-5''); 2.23 (3H, H-2', H-2'' and CH<sub>2</sub>C≡); 1.63 (2H, p, CH<sub>2</sub>); 1.47 (1H, t, ≡CH); 1.43 (2H, p, CH<sub>2</sub>).

### Example 8

- 5 The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3 {tetramethyl 2,2',2'', 2'''-[(4-(hex-5-yn-1-yl)pyridine-2,6-diyl)bis(methylene-nitrilo)]tetrakis(acetato)-uridine (7) — Method B

Compound 9 was coupled to compound 5 using the method described in example 5. The yield was 60 %. The product was spectroscopically and chromatographically  
10 identical with the material synthesized in example 6.

### Example 9

The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3-{6-{2,6-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]pyridin-4-yl}hex-5-yn-1-yl}uridine 3'-[*O*-(2-cyanoethyl)-N,N-diisopropyl]phosphoramidite (8)

- 15 Compound 7 was phosphitylated using the method described in example 2. Purification was performed on silica gel (eluent CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N:MeOH 90:10:5; v/v/v). Compound 8: <sup>31</sup>P NMR (CDCl<sub>3</sub>): δ 149.4 (0.5 P), 149. 1 (0.5 P).

### Example 10

The synthesis of 6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hexan-1-ol (10)

- 20 To a stirred solution of 6-aminohexan-1-ol (0.50 g, 4.27 mmol) in dichloromethane (10 ml) was added dropwise a solution of dabsyl chloride (0.5 g, 1.54 mmol) in dichloromethane (10 ml). After 1h the mixture was washed with sat aq. NaHCO<sub>3</sub>. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. Purification on silica



gel (eluent CH<sub>2</sub>Cl<sub>2</sub> containing 1 % (v/v) MeOH) yielded the title compound as red solid. Compound **10**: <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.97-7.89 (6H, m); 6.76 (2H, d, *J* 9.3); 4.50 (1H, br t, *J* 6.3); 3.60 (2H, t, *J* 6.2); 3.13 (6H, s); 2.98 (2H, q, *J* 6.9); 1.63 (1H, br); 1.50 (4H, m); 1.30 (4H, m).

## 5 Example 11

The synthesis of *N*3-[6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hex-1-yl-5'-*O*-(4,4'-dimethoxytrityl)thymidine (**11**)

The title compound was synthesized by Mitsunobu alkylation of 5'-*O*-(4,4'-dimethoxytrityl)thymidine and compound **10** using procedures described in example 1. The yield was 74 %. Compound **11**: <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): d 7.98-7.88 (6H, m, dabsyl); 7.57 (1H, *J* 1.2 H-6); 7.40 (2H, d, DMT); 7.30-7.24 (7H, m, DMT); 6.83 (4H, d, *J*, 9.0, DMT), 6.75 (2H, d, *J* 7.3, dabsyl); 6.48 (1H, dd, *J* 7.8), 5.03 (1H, t, *J* 6.1, NH); 4.55 (1H, m, H-3'); 4.07 (1H, m, H-4'); 3.92 (2H, m, NCH<sub>2</sub>); 3.79 (6H, s, 2 · OCH<sub>3</sub>); 3.46 (1H, dd, *J* 3.2 and 10.5, H-5'); 3.36 (1H, dd, *J* 2.9 and 10.5, H-5''); 3.12 (6H, s, N(CH<sub>3</sub>)<sub>2</sub>); 2.99 (2H, m, CH<sub>2</sub>NH); 2.46 (1H, m, H-2''); 2.31 (1H, m, H-2' and 3'-OH); 1.59 (4H, m, 2 · CH<sub>2</sub>); 1.38-1.25 (4H, m, 2 · CH<sub>2</sub>).

## Example 12

The synthesis of *N*3-[6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hex-1-yl-5'-*O*-(4,4'-dimethoxytrityl)thymidine 3'-*O*-(2-cyanoethyl *N,N*-diisopropyl) phosphoramidite (**12**)

Phosphitylation of compound **11** as described in example 3 yielded the title compound as a solid (purification on silica gel using the eluent described in example 9).

**Example 13**

The synthesis of tritylethoxyethanol (**13**)

Bisethoxyethanol (10 ml) was dried by coevaporation with dry pyridine and dissolved in the same solvent (20 ml). Trityl chloride was added and the mixture  
 5 was stirred 2 h at ambient temperature. The solvent was evaporated in vacuo. The residue was dissolved in methylene chloride, washed with sat. NaHCO<sub>3</sub>, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. Precipitation from ethyl ether yielded the title compound as a white powder. It was used in the next step without further characterization.

**10 Example 14**

The synthesis of 4-iodophenoxyethoxyethanol (**14**)

Compound **13** was allowed to react with 4-iodophenol as described in example 1. When the reaction was completed (ca. 2 h) the solvent was evaporated off and the residue was suspended in diethyl ether and passed through a short column of silica  
 15 gel. The eluent was removed in vacuo and the residue was dissolved in the mixture of TFA and ethanol (9:1, v/v) and stirred overnight at ambient temperature after being concentrated. The residue was taken in methylene chloride and washed with sat. NaHCO<sub>3</sub>, dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. Purification was performed on silica gel. The column was eluted initially with methylene chloride to remove trityl  
 20 carbinol and then with the mixture of CH<sub>2</sub>Cl<sub>2</sub>:MeOH (9:1, v/v) to elute the desired product. Compound **14**: <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.53 (2H, d, *J* 9.0); 6.68 (2H, d, *J* 9.0); 4.07 (2H, m); 3.83 (2H, m); 3.73 (2H, m); 3.64 (2H, m); 2.26 (1H, br).

**Example 15**

The synthesis of tetramethyl 2,2',2'',2'''-{6,6'-[4'-hydroxyethoxyethoxyphenoxy-  
 25 ethynyl]pyridine-2,6-diyl}bis(methylene-nitrilo)tetrakis(acetate) (**16**)

Compound **14** was allowed to react with tetramethyl 2,2',2'',2'''-[4-ethynyl-pyridine-2,6-diyl]bis(methylenenitrilo)tetrakisacetate **15** using the reaction described in example 5, but reaction was completed in 5 h at ambient temperature. Compound **15**:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.53 (2H, s); 7.49 (2H, d,  $J$  8.8); 6.98 (2H, d,  $J$  8.8); 4.17 (2H, m); 4.03 (4H, s); 3.89 (2H, m); 3.79 (2H, m); 3.72 (12H, s); 3.68 (2H, m); 3.64 (8H, s); 2.38 (1H, br).

### Example 16

The synthesis of 5'-*O*-(4,4'-dimethoxytrityl)-*N*3-{tetramethyl-2,2',2'',2'''-{6,6'-[4'-ethoxyethoxyphenoxyethynyl]pyridine-2,6-diyl}bis(methylenenitrilo)-tetrakis(acetato)}thymidine (**17**)

Compound **16** was allowed to react with 5'-*O*-(4,4'-dimethoxytrityl)thymidine using the reaction described in example 1. Compound **17**:  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  7.59 (1H, s); 7.51 (2H, d,  $J$  8.8); 7.47 (2H, s); 7.49-7.23 (9H, DMT); 6.99 (2H, d,  $J$  8.8); 6.86 (4H, d, DMT); 6.23 (1H, t,  $J$  6.8); 5.34 (1H, d,  $J$  4.2; exch. with  $\text{D}_2\text{O}$ ); 4.32 (1H, m); 4.10 (2H, m); 4.01 (2H, m); 3.92 (1H, m); 3.90 (4H, s); 3.74 (2H, m); 3.71 (12H, s); 3.62 (2H, m); 3.61 (8H, s); 3.10 (2H, m); 2.22 (2H, m); 1.47 (3H, s).

### Example 17

The synthesis of 2,2'-(4-iodo-1H-pyrazol-1,3-diyl)bis(pyridine) 1,1'-dioxide (**19**)

To a stirred solution of 2,2'-(1H-pyrazol-1,3-diyl)bis(pyridine) 1,1'-dioxide (9.9 g, 38.9 mmol) in conc. nitric acid /water 25 ml (1:1, v/v) iodine (9.8 g, 38.9 mmol) was added and the mixture was heated overnight at 95 °C. The mixture was allowed to cool to room temperature and alkalized with 1 M NaOH. The aqueous layer was extracted three times with  $\text{CHCl}_3/\text{EtOH}$  (4:1), dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated. Purification on silica gel (eluent  $\text{CH}_2\text{Cl}_2:\text{MeOH}$ , 9:1, v/v) yielded 12.2 g (82 %) of

19.  $^1\text{H}$  NMR  $\delta$  ( $\text{CDCl}_3$ ) 9.52 (1H, s); 8.37 (1H, m); 8.34 (1H, m); 8.09 (1H, m); 7.51 (1H, m); 7.41-7.30 (3H, m); 7.25 (1H, m).

### Example 18

The synthesis of 6,6'-(4-Iodo-1*H*-pyrazole-1,3-diyl)bis(pyridine)-2,2'-dicarbonyl nitrile (**20**)

Trimethylsilyl cyanide (16.2 ml, 0.13 mol) was added to a mixture of **19** (4.92 g, 13.0 mmol) and  $\text{CH}_2\text{Cl}_2$  (133 ml). After 5 min, benzoyl chloride (6 ml, 52 mmol) was added, and the mixture was stirred for 24 h at ambient temperature. The mixture was then concentrated (to *ca.* 15 ml), 10 %  $\text{K}_2\text{CO}_3$  solution (130 ml) was added and the mixture stirred for 2 h. A cold mixture was filtered, and the main product fraction was washed with water (50 ml) and cold  $\text{CH}_2\text{Cl}_2$  (2 x 20 ml). The organic phase of filtrate was separated, and evaporated to dryness. A cooled mixture of the residue and diethyl ether (200 ml) was filtered. Total yield was 4.36 g (85 %); IR (KBr)  $2237\text{ cm}^{-1}$  ( $\text{C}\equiv\text{N}$ ),  $1590$ ,  $1574\text{ cm}^{-1}$  (arom.);  $^1\text{H}$  NMR  $\delta$  ( $\text{CDCl}_3$ ) 8.79 (1H, s); 8.30 (1H, dd,  $J$  0.8 and 3.9); 8.34 (1H, dd,  $J$  0.8 and 3.5); 8.02 (1H, dd,  $J$  7.5 and 8.5); 7.95 (1H, dd,  $J$  7.7 and 8.1); 7.73 (1H, dd,  $J$  1.0 and 7.7); 7.66 (1H, dd,  $J$  0.8 and 7.5).

### Example 19

The synthesis of tetramethyl 2,2',2'',2'''-[[6,6'-(4-Iodo-1*H*-pyrazole-1,3-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)]tetrakis(acetate) (**22**)

A suspension of compound **20** (5.06 g, 12.7 mmol) and dry tetrahydrofuran (140 ml) was deaerated with nitrogen. Borane in tetrahydrofuran (1 M, 140 ml) was added within 10 min into the reaction mixture. After stirring for 24 h at room

temperature, excess borane was destroyed by addition of MeOH, the mixture was evaporated and the residue dissolved in MeOH saturated with dry HCl (20 ml). After stirring for 1 h, the mixture was evaporated, and the residue treated with tetrahydrofuran (20 ml). The cooled mixture was filtered and the solid material washed with cold tetrahydrofuran (10 ml) and diethyl ether (5 ml). To give 7.47 g (94 %) [ms (FAB<sup>+</sup>) 407] of crude compound **21**. A mixture of this material (3.1 g, 5.3 mmol), BrCH<sub>2</sub>COOMe (2.0 ml, 21.1 mmol), dry *N,N*-diisopropylethylamine (13.8 ml, 79 mmol) and dry acetonitrile (110 ml) was refluxed for 24 h. After evaporation, the residue was dissolved in CHCl<sub>3</sub> (50 ml), washed with water (3 x 25 ml) and dried with Na<sub>2</sub>SO<sub>4</sub>. The product was purified by flash chromatography [silica gel, petroleum ether (40–60°)/ethyl acetate, 1:1]; yield 56 % of compound **22**. IR (film) 1732 cm<sup>-1</sup> (C=O), 1144 cm<sup>-1</sup> (C-O); <sup>1</sup>H NMR δ(CDCl<sub>3</sub>) 1.47 (18 H, s), 1.48 (18 H, s), 3.53 (4 H, s), 3.57 (8 H, s), 4.06 (2 H, s), 4.15 (2 H, s), 7.54 (1 H, d, *J* = 7.6 Hz), 7.72 (1 H, d, *J* = 8.0 Hz), 7.79 (1 H, t, *J* = 7.6 Hz), 7.82 (1 H, t, *J* = 8.0 Hz), 7.91 (1 H, d, *J* = 7.6 Hz), 7.95 (1 H, d, *J* = 8.0 Hz), 8.70 (1 H, s).

### Example 20

The synthesis of tetramethyl 2,2',2'',2'''-{[6,6'-(4-(5-hydroxyhexyn-1-yl)-1*H*-pyrazole-1,3-diyl)bis(pyridine)-2,2'-diyl]bis(methylenenitrilo)}tetrakis(acetate) (**23**)

A mixture of compound **22** (1.0 g, 1.44 mmol), 5-hexyn-1-ol (0.19 ml, 1.72 mmol), dry piperidine (4.5 ml) and dry DMF (6 ml) was deaerated with argon. Bis(triphenylphosphine)palladium(II) chloride (20 mg, 29 μmol) and copper iodide (11 mg, 58 μmol) was added and the mixture was stirred for 12 h at 40° C. After evaporation, the residue was dissolved in CHCl<sub>3</sub> (90 ml), washed with water (3 x 45 ml) and dried with Na<sub>2</sub>SO<sub>4</sub>. The product was purified by flash chromatography (eluent CH<sub>2</sub>Cl<sub>2</sub>:MeOH, 9:1). Yield, 0.90 g. [M+H]<sup>+</sup> 665.

**Example 21**

The synthesis of tetramethyl 2,2',2'',2'''-[6,6'-(4-(hexan-6-ol)-1*H*-pyrazole-1,3-diyl)bis(pyridine)-2,2'-diyl]bis(methylene-nitrilo)} tetrakis(acetate) (**24**)

A mixture of compound **23** (0.45 g, 0.68 mmol) 10 % Pd on carbon (50 mg) and  
 5 MeOH (30 ml) was stirred in a hydrogen atmosphere for 2.5 h. After filtration, the filtrate was evaporated and the residue was purified by flash chromatography (CH<sub>2</sub>Cl<sub>2</sub>:MeOH, 9:1). The yield was 350 mg, 77 %; ms 669 [M+H]<sup>+</sup>

**Example 22**

The synthesis of the non-nucleosidic phosphoramidite (**25**)

10 Compound **24** was phosphitylated using the method described in example 3. Yield after silica gel column chromatography (CH<sub>2</sub>Cl<sub>2</sub>:MeOH:TEA 9:1:1; v/v/v). <sup>31</sup>P NMR (CDCl<sub>3</sub>): 147.8.

**Example 23**

The synthesis of 5'-*O*-(4,4'-dimethoxytrityl)-*N*3-{tetramethyl-2,2',2'',2'''-[6,6'-(4'-hydroxyethoxyethoxyphenyl-ethynyl)pyridine-2,6-diyl]bis(methylene-nitrilo)} tetrakis(acetato)} thymidine 3'-succinate (**26**)  
 15

Compound **17** (0.67 mmol) was dissolved in dry pyridine (5 ml). Succinic anhydride (135 mg, 1.35 mmol) and cat. amount of DMAP were added, and the mixture was stirred overnight at room temperature and concentrated. The residue  
 20 was dissolved in dichloromethane, washed with aqueous triethylamine and dried. Purification was performed on silica gel (eluent:10% MeOH in dichloromethane). Compound **26**: <sup>1</sup>H NMR (DMSO-d<sub>6</sub>): δ 7.59 (1H, s); 7.54 (2H, d, *J* 8.8); 7.50 (2H, s); 7.38-7.21 (9H, DMTr); 7.00 (2H, d *J* 8.8); 6.90 (4H, d, DMTr); 6.27 (1H, dd); 5.31 (1H, m); 4.11 (3H, m); 4.02 (2H, m); 3.91 (4H, s); 3.79 (2H, m); 3.73 (8H, s);

3.61 (2H, m); 3.60 (12H, s); 3.37 (2H, m); 2.67 (2H, t); 2.42 (2H, t); 2.22 (2H, m); 1.47 (3H, s).

#### Example 24

The synthesis of the solid support (**27**)

- 5 Long chain alkylamine controlled pore glass was treated with a mixture of 10 % TEA in 80 % aqueous ethanol, washed with acetonitrile and dried. Compound **23** (0.5 mmol; as a pyridinium salt), *N,N'*-diisopropylcarbodiimide (1.0 mmol, 157  $\mu$ l); and *N*-hydroxysuccinimide (0.5 mmol, 58 mg) was added to a suspension of the solid support in dry pyridine (5 ml) and the mixture was shaken overnight at ambient temperature. The suspension was filtered, washed with dry pyridine, kept in a mixture of Ac<sub>2</sub>O:pyridine:*N*-methylimidazole (1:5:1; v/v/v) for 10 min, and finally washed with ether. Loading as judged on DMTr cation assay was 34  $\mu$ molg<sup>-1</sup>.
- 10

#### Example 25

- 15 The synthesis of (E)-3-(4'-Bromophenyl)-1-pyrid-2'-yl)prop-2-enone (**28**)

4-Bromobenzaldehyde (50 g, 0.27 mol) was added in the ice-cold mixture methanol (540 ml) and water (110 ml) containing potassium hydroxide (15.2 g). After all aldehyde was dissolved 2-acetylpyridine (30.3 ml, 0.27 mol) was added and the reaction was allowed to proceed overnight at ambient temperature. The precipitation formed was filtered, washed with cold methanol and dried. Yield was 64 g (82%).  
 1H NMR (CDCl<sub>3</sub>):  $\delta$  8.75 (1H, br. d); 8.31 (1H, d, *J* 12 Hz); 8.20 (1H, br d); 7.90 (1H, m); 7.87 (1H, d, *J* 12); 7.59 (5H, m). MS (EI+) 288, 289 [M+].

- 20

**Example 26**

The synthesis of 4''-(4'''-bromophenyl)-2,2':6',2''-terpyridine (**30**)

A mixture of compound **28** (20.6 g, 71 mmol), dry ammonium acetate (137 g) and freshly prepared N-[2-(pyrid-2'-yl)-2-oxo-ethyl]pyridinium iodide (**29**; 23.3 g, 71 mmol) in dry methanol (650 ml) was heated at reflux overnight. The mixture was cooled to room temperature and refrigerated. The precipitation was separated by filtration, washed with cold methanol and dried. Yield was 12.5 g (45%). <sup>1</sup>H NMR (dmsO-d<sub>6</sub>) δ: 8.77 (2H, br d, *J* 4); 8.71 (2H, s); 8.69 (2H, d *J* 7.9); 8.06 (2H, td, *J* 2.5 and 7.5); 7.92 (2H, d, *J* 7.5); 7.79 (2H, d, *J* 7.5); 7.55 2H, m). MS (EI+) 388, 390 [M+].

**Example 27**

The synthesis of 4''-(4'''-Bromophenyl)-2,2':6',2''-terpyridine N,N''-Dioxide (**31**)

3-Chloroperbenzoic acid (29.1 g, 121 mmol) was added to compound **30** (12.4 g, 32 mmol) in dichloromethane (500 ml) and the mixture was stirred overnight at ambient temperature. The mixture was washed with 10 % sodium carbonate (300 ml), dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. Purification on silica gel (eluent 10% methanol in dichloromethane) gave 11.4 g (85%) of product. <sup>1</sup>H NMR (dmsO-d<sub>6</sub>) δ: 9.06 (2H, s); 8.43 (2H, m); 8.24 (2H, m); 7.80 (4H, s); 7.54 (4H, m). MS (EI+) 419, 421 [M+].

**Example 28**

The synthesis of 4''-(4'''-Bromophenyl)-2,2':6',2''-terpyridine-6,6''-dicyanitrile (**32**)

Trimethylsilylcyanide (13.7 ml, 110 mmol) was added to compound **31** (4.6 g, 11 mmol) in dichloromethane (170 ml). After 5 min, benzoyl chloride (5.1 ml, 44 mmol) was added within 20 min. After stirring overnight, the mixture was



evaporated to half volume, 10% solution of  $K_2CO_3$  (100 ml) was added, the mixture was stirred for 15 min, and the precipitate filtered and washed with water and cold dichloromethane. Yield was 3.69 g (77%).  $^1H$  NMR (dms $o$ - $d_6$ ):  $\delta$  8.98 (2H, d,  $J$  8.0); 8.68 (2H, s); 8.31 (2H, t,  $J$  7.6); 8.21 (2H, d,  $J$  7.6); 7.97 (2H, d,  $J$  8.4); 7.80 (2H, d,  $J$  8.4). IR (KBr): 2237  $cm^{-1}$  (CN). MS (EI+): 437, 439  $[M^+]$ .

### Example 29

The synthesis of tetramethyl 2,2',2'',2'''-{[4'-(4''-bromophenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis(methylenenitrilo)}tetrakis (acetate) (**34**)

A suspension of compound **32** (3.65 g, 8.3 mmol) in dry THF (100 ml) was deaerated with argon.  $BH_3 \cdot THF$  was added during 20 min. After stirring for 2.5 h at ice-bath, the excess of borane was destroyed by addition of methanol. The mixture was evaporated, and the residue was dissolved in methanol saturated with HCl (50 ml). After stirring for 2 h at room temperature, the mixture was concentrated. The residue was suspended in THF, filtered, washed with THF and dried. This material was suspended in dry DMF (50 ml). Diisopropylethylamine (21 ml), methyl bromoacetate (3.1 ml, 33.3 mmol) and KI (1.51 g, 9.1 mmol) were added, and the mixture was stirred overnight at room temperature and concentrated. The residue was dissolved in dichloromethane (80 ml), washed with sat  $NaHCO_3$  (3  $\cdot$  40 ml) and dried. Purification was performed on silica gel (eluent pet. ether: ethyl acetate:triethylamine 5:2:1, v/v/v) Yield was 6.6 g.  $^1H$  NMR ( $CDCl_3$ )  $\delta$ : 8.68 (2H, s); 8.55 (2H, d,  $J$  6); 7.87 (2H, t,  $J$  6); 7.81 (2H, d,  $J$  6); 7.68 (2H, d,  $J$  6); 7.62 (2H, d,  $J$  6); 4.19 (4H, s); 3.73 (8H, s); 3.70 (12H, s).

### Example 30

The synthesis of tetramethyl 2,2',2'',2'''-{[4'-(4''-(6-hydroxy-2-hexyn-1-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis (methylenenitrilo)}tetrakis(acetate) (**35**)

Compound **34** (2.0 g, 2.72 mmol) and 5-hexyn-1-ol (360 ml; 3.28 mmol) were dissolved in the mixture of dry THF (15 ml) and triethylamine (4 ml) and the mixture was deaerated with argon for 10 min. Pd(Ph<sub>3</sub>P)<sub>2</sub>Cl<sub>2</sub> (37.5 mg, 0.053 mmol) and CuI (21.9 mg, 0.11 mmol) were added and the mixture was stirred overnight at 60 °C. The cooled mixture was filtered and the filtrate was concentrated in vacuo. The residue was dissolved in dichloromethane (50 ml), washed with water (2 · 20 ml) and dried. Purification on silica gel (eluent 10% methanol in dichloromethane (v/v)) gave 1.63 g (80%) of product. IR (film) 2232 cm<sup>-1</sup> (C≡C, weak). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 8.70 (2H, s); 8.55 (2H, d, *J* 7.9); 7.87 (2H, t, *J* 7.9); 7.85 (2H, d, *J* 8.6); 7.61 (2H, d, *J* 7.6); 7.56 (2H, d, *J* 8.2); 4.19 (4H, s); 3.73 (8H, s); 3.75 (2H, m); 3.70 (12H, s); 2.52 (2H, t, 6.7); 1.77 (6H, m); 1.74 (1H br). MS (FAB+) 752.

### Example 31

The synthesis of 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-3-(2,2',2'',2'''-{[4'-(4''-(5-hexyn-6-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis (methylenenitrilo)} tetra-kis(acetato) uridine (**36**)

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)uridine was allowed to react with compound **35** under Mitsunobu conditions as described for compound **1**. Purification was performed on silica gel (eluent petr. ether: ethyl acetate: triethylamine; 2:5:1; v/v/v). Yield was 61%. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 8.70 (2H, s.); 8.55 (2H, d); 7.86 (4H, m); 7.76 (1H, d); 7.57 (4H, m); 7.37 (nH, d); 6.83 (4H, d); 6.37 (1H, t); 5.45 (1H, d); 4.59 (1H, m); 4.21 (4H, s); 4.09 (1H, m); 3.99 (2H, t); 3.79 (8H, s); 3.70 (12H, s); 3.49 (2H, m); 2.79 (1H, br s); 2.53 (2H, m and t); 2.29 (1H, m); 1.78 (4H, m).

**Example 32**

The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3-6-{ {4-{6,6''-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]-2,2':6',2''-terpyridine-4'-yl}phenyl}hex-5-yn-1-yl}uridine 3'-[*O*-(2-cyanoethyl)-N,N-diisopropyl]phosphoramidite (**37**)

- 5 Phosphitylation of compound 36 using the method described in Example 3 yielded the title compound after silica gel column chromatography as a white powder <sup>31</sup>P NMR (CDCl<sub>3</sub>): δ 148.7 (0.5 P), 148.3 (0.5 P).

**Example 33**

- 10 6-{4-{6,6''-bis[N,N-bis(methoxycarbonylmethyl)aminomethyl]-2,2':6',2''-terpyridine-4'-yl}phenyl}hex-5-yn-1-ol [*O*-(2-cyanoethyl)-N,N-diisopropyl]-phosphoramidite (**38**).

Phosphitylation of compound 30 yielded the title compound as a Colorless oil (purified on silica gel) <sup>31</sup>P NMR (CDCl<sub>3</sub>): δ 147.7 (1P).

**Example 34**

- 15 Introduction of primary amino groups to the oligonucleotide structure with the aid of compound **3** — Labeling of the amino groups with an europium(III) chelate

- 20 A model sequence d(TTCCTCCACTGT) was synthesized on an ABI instrument, and 5 phosphoramidites **3** were coupled to its 5'-terminus using standard conditions (concentration 0.1 M in acetonitrile, coupling time 30 s): No difference in coupling efficiency between **3** and normal nucleosidic building blocks were detected as judged on DMTr-cation response. After standard ammoniolytic deprotection, the oligonucleotide prepared was isolated on PAGE and desalted on NAP columns. This oligonucleotide was finally labeled with the non-luminescent europium(III) chelate (**39**) as described in Dahlén, P., Liukkonen, L., Kwiatkowski, M., Hurskainen, P.,

Itiä, A., Siitari, H., Ylikoski, J., Mukkala, V.-M., and Lövgren, T., *Bioconjugate Chem.*, **1994**, 5, 268.

### Example 35

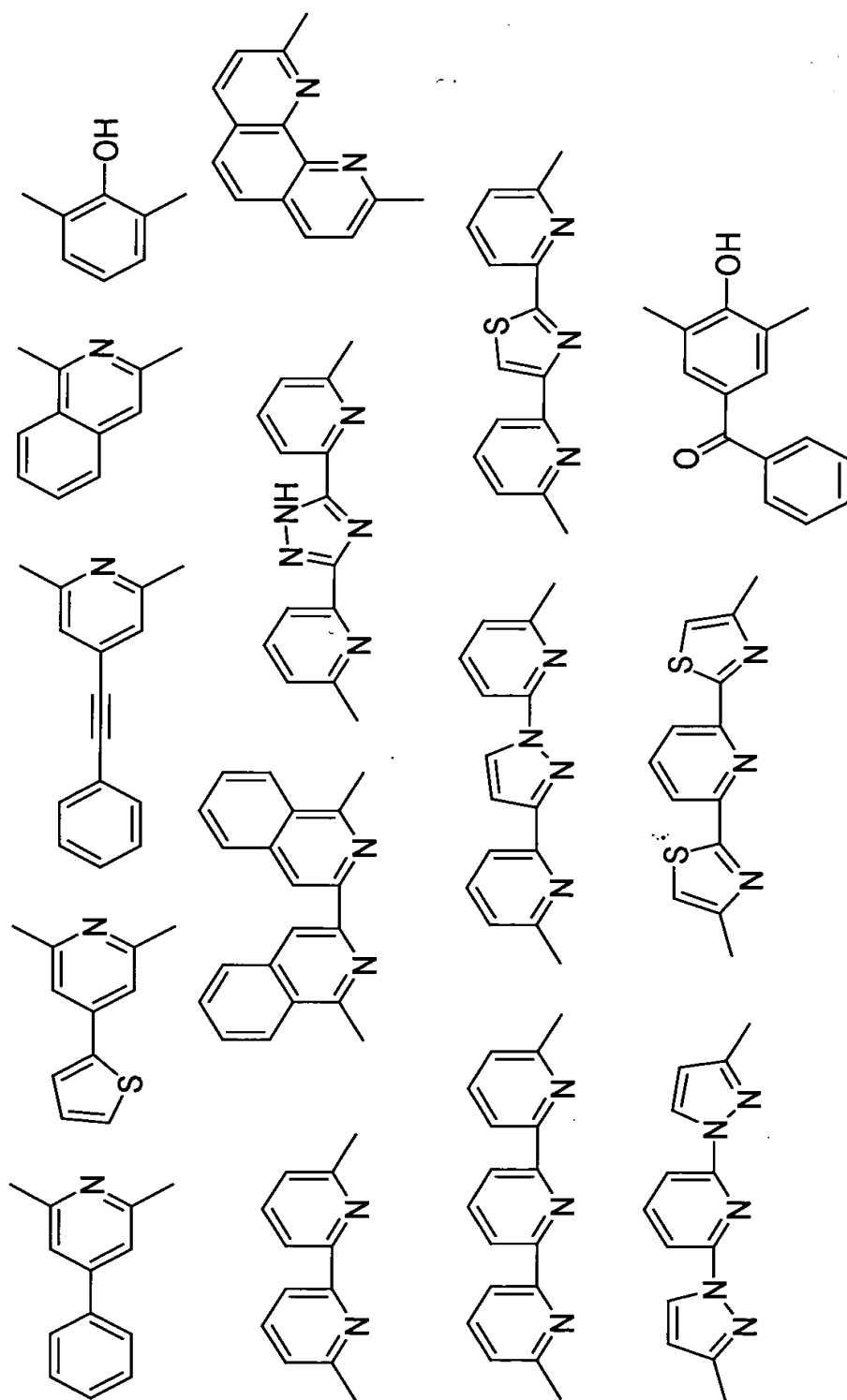
Introduction of lanthanide(III) chelates to the oligonucleotide structure with the aid  
5 of compound **8**

Model sequences were synthesized as described above in Example 34. One or 10  
phosphoramidites **8** were coupled to its 5'-terminus using standard conditions. No  
difference in coupling efficiency between **8** and normal nucleosidic building blocks  
were detected. When the chain assembly was completed, the oligonucleotides were  
10 deprotected by first treating the solid support with 0.1 M sodium hydroxide for 4 h  
at ambient temperature. 1.0 M ammonium chloride was then added, and the solution  
was concentrated in vacuo. The residue was treated with conc. ammonia for 16 h at  
60 °C, after which europium citrate (10 eq. per ligand) was added, and the mixture  
was kept 90 min at room temperature. Desalting by NAP followed by RP HPLC  
15 yielded the desired oligonucleotide conjugates containing one or ten europium(III)  
chelates in their structure.

### Example 36

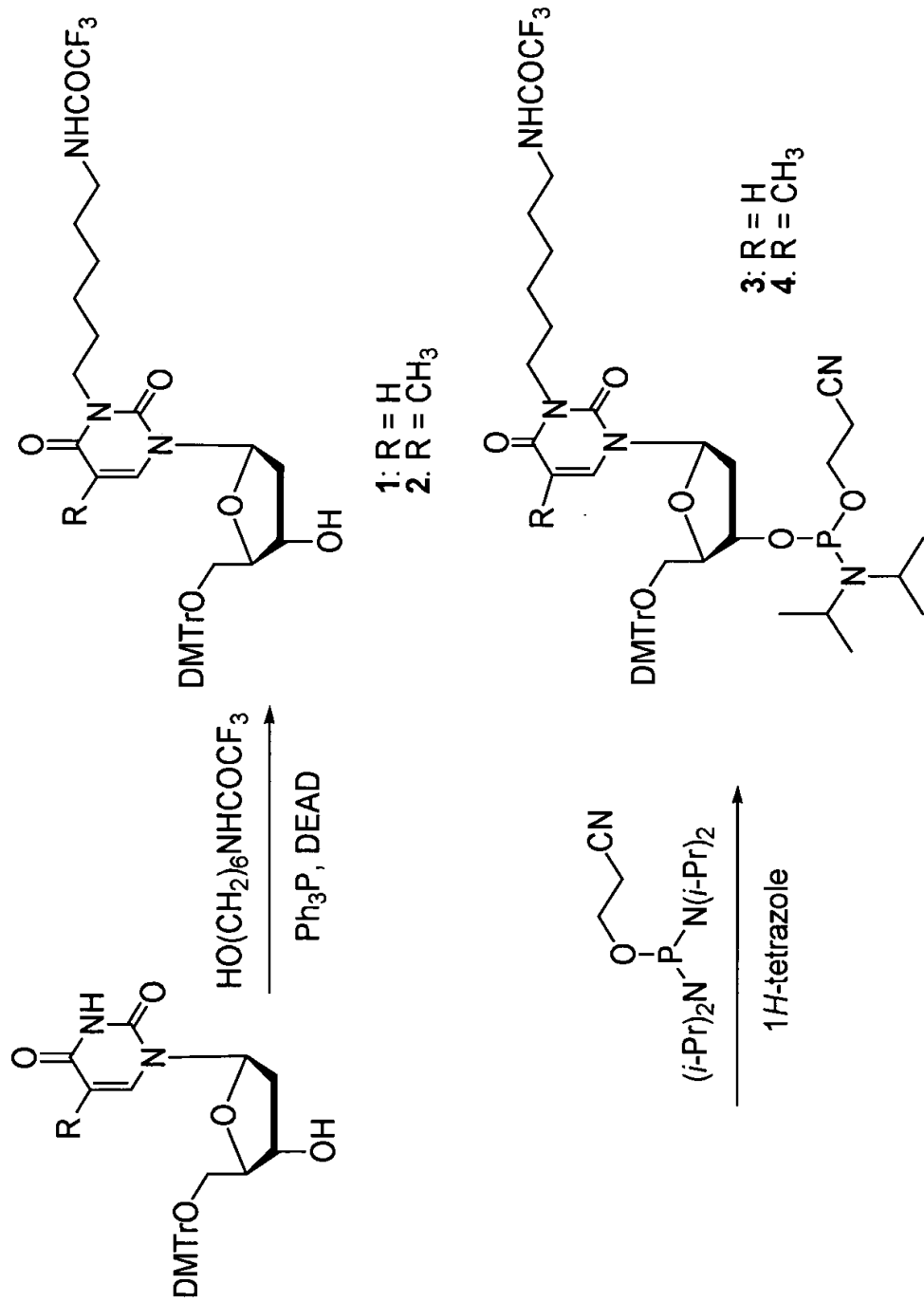
Introduction of a lanthanide(III) chelate to the oligonucleotide structure with the aid  
of compound **38**

20 The luminescent terpyridine chelate was introduced to the 5'-terminus of the  
oligonucleotide structure in the aid of blocks **38** analogously as described for block  
**8**, except DMTr-On synthesis was applied.

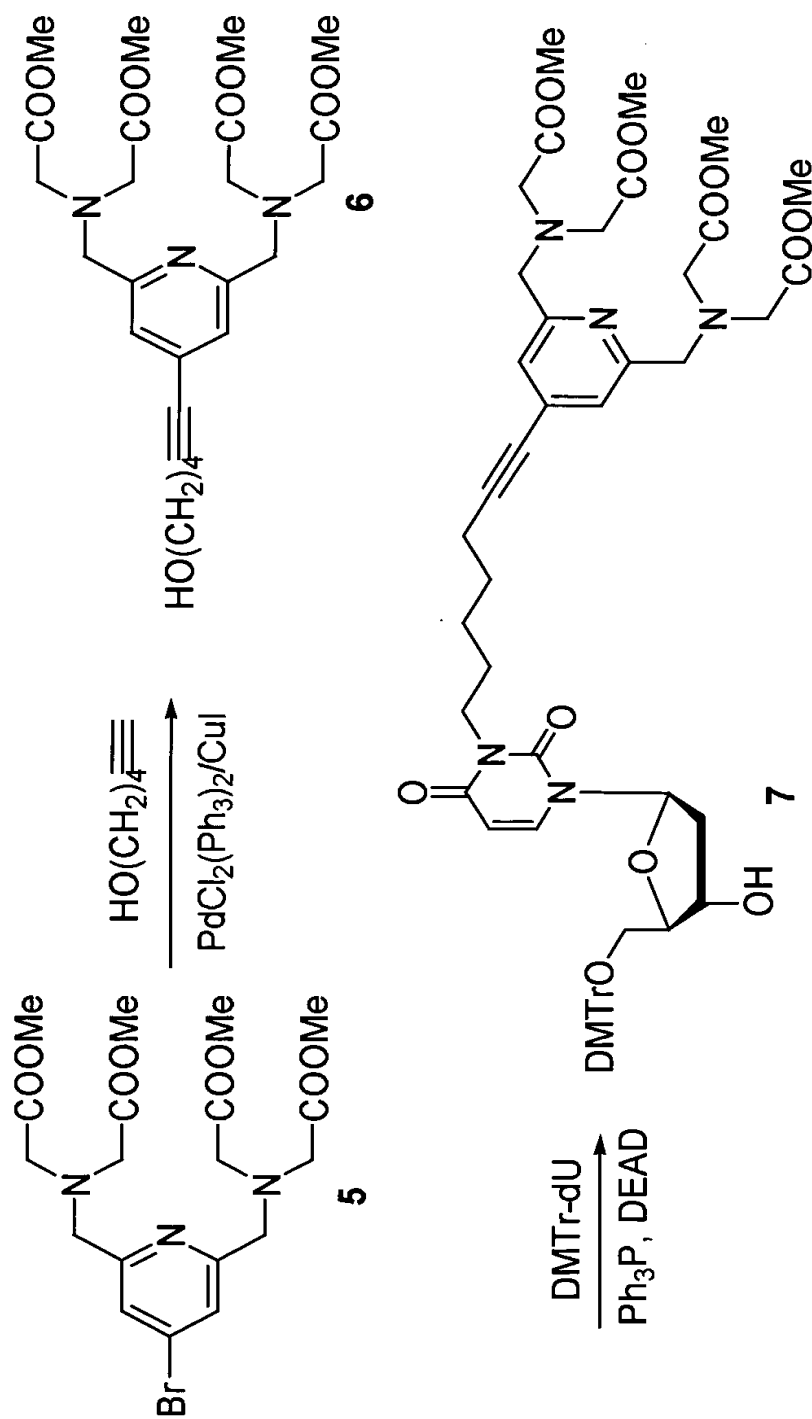


## SCHEME 1A





SCHEME 2



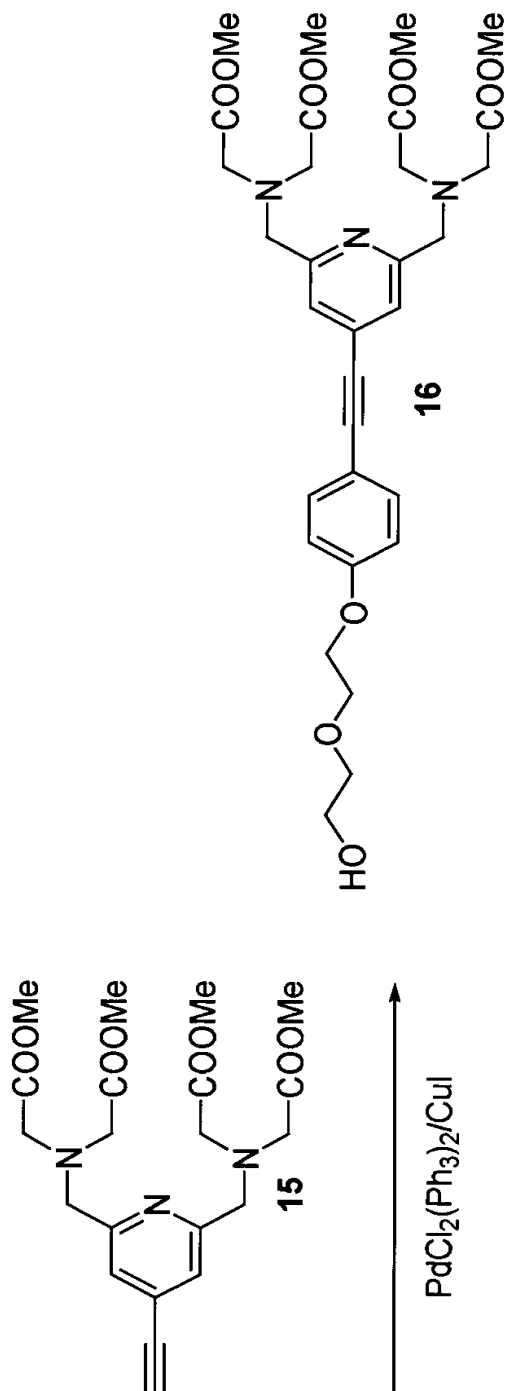
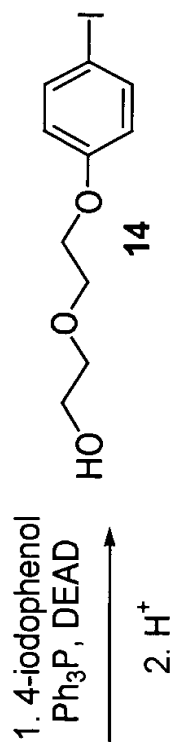
SCHEME 3A



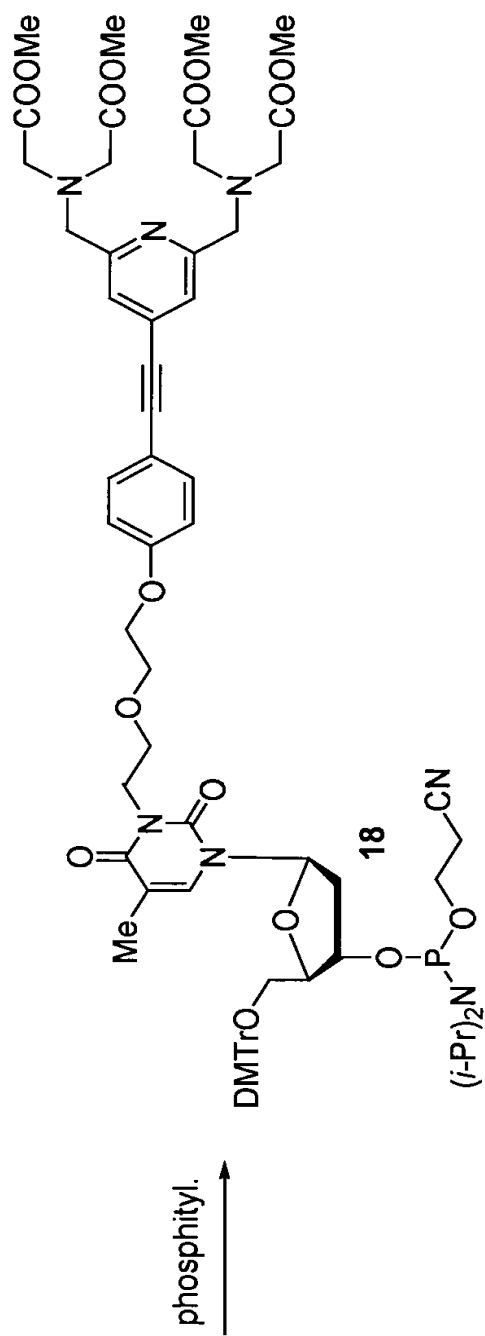
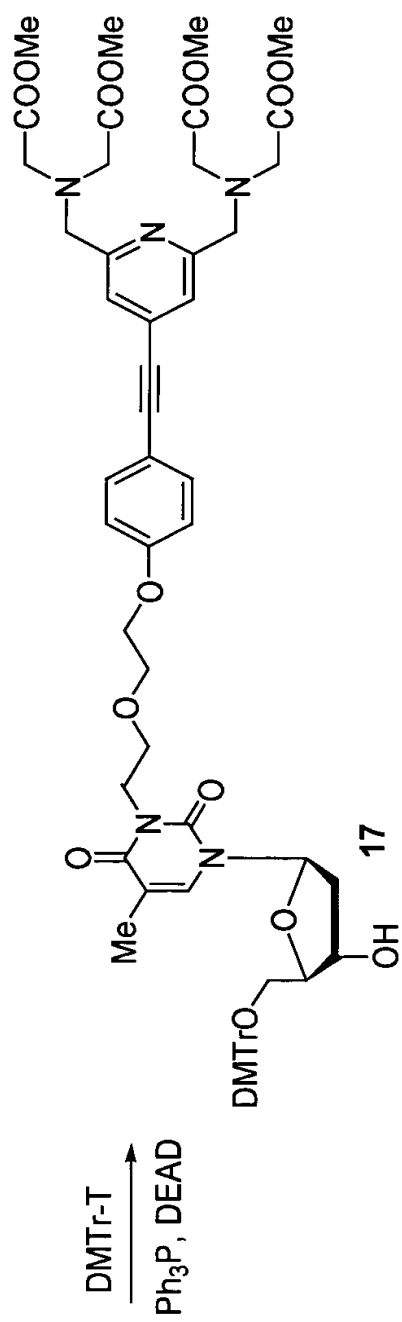




## SCHEME 4



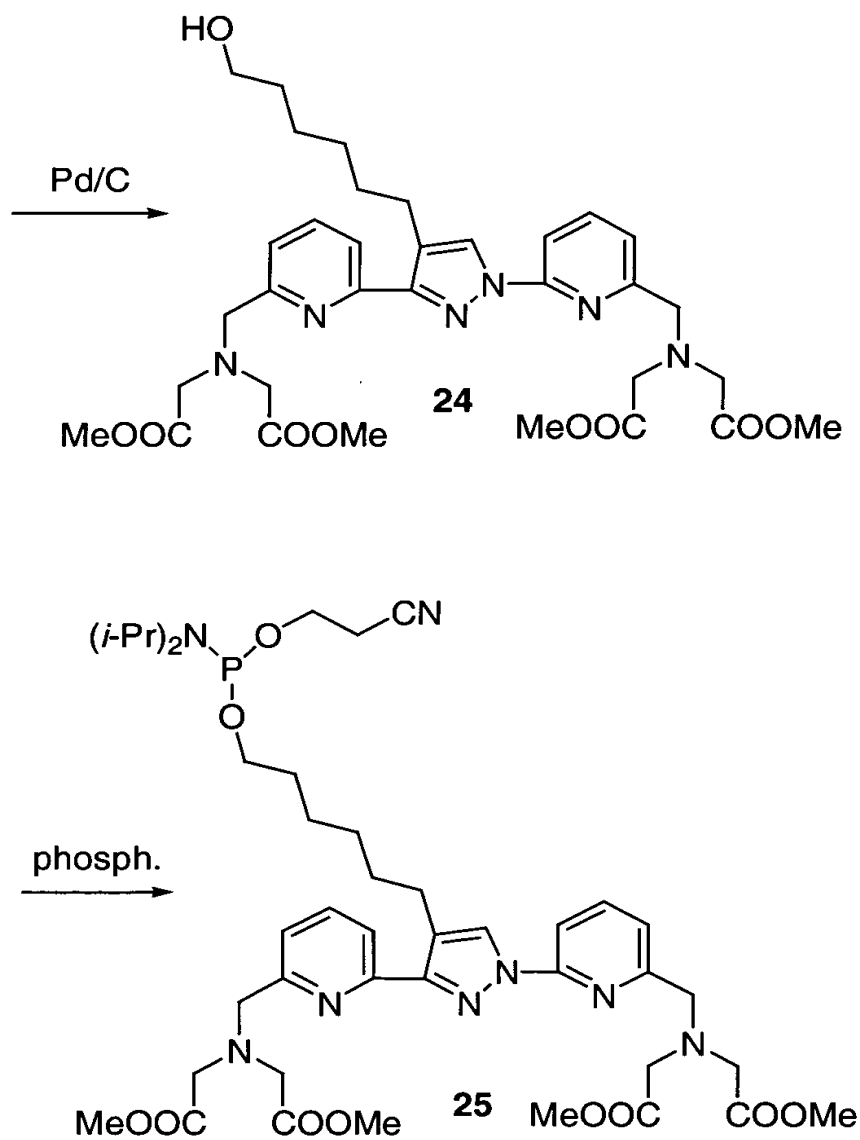
## SCHEME 5A



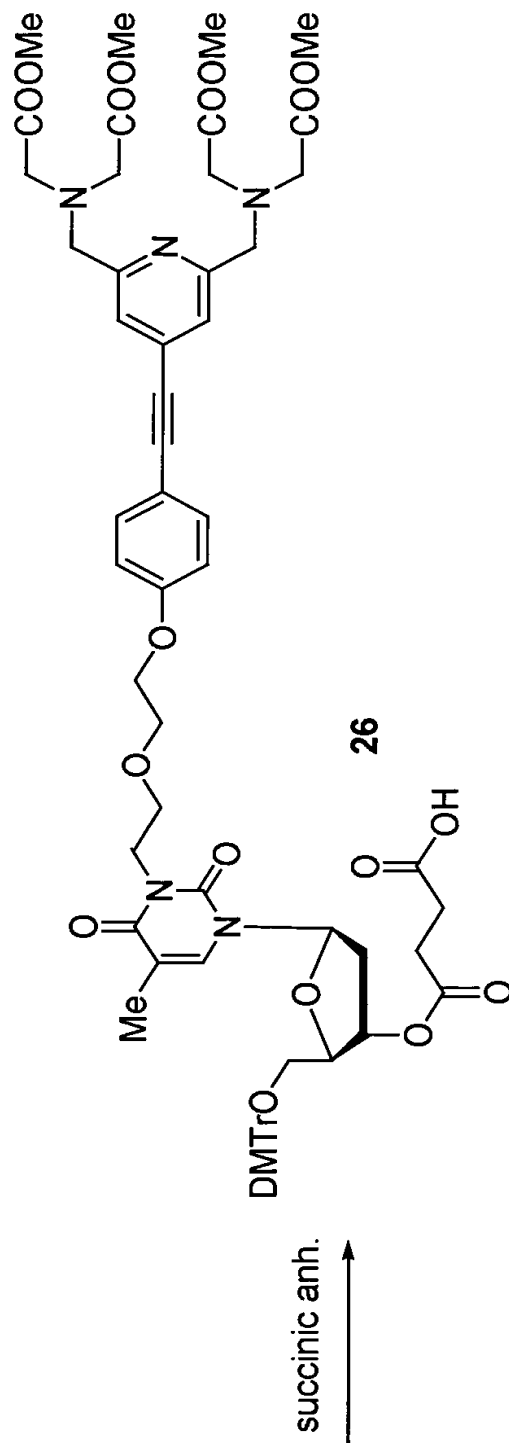
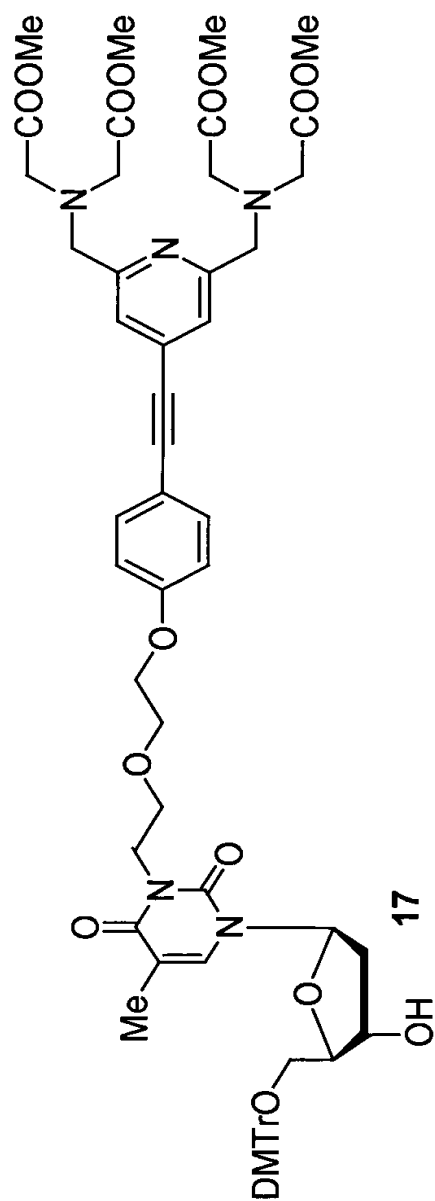
### SCHEME 5B



### SCHEME 6A



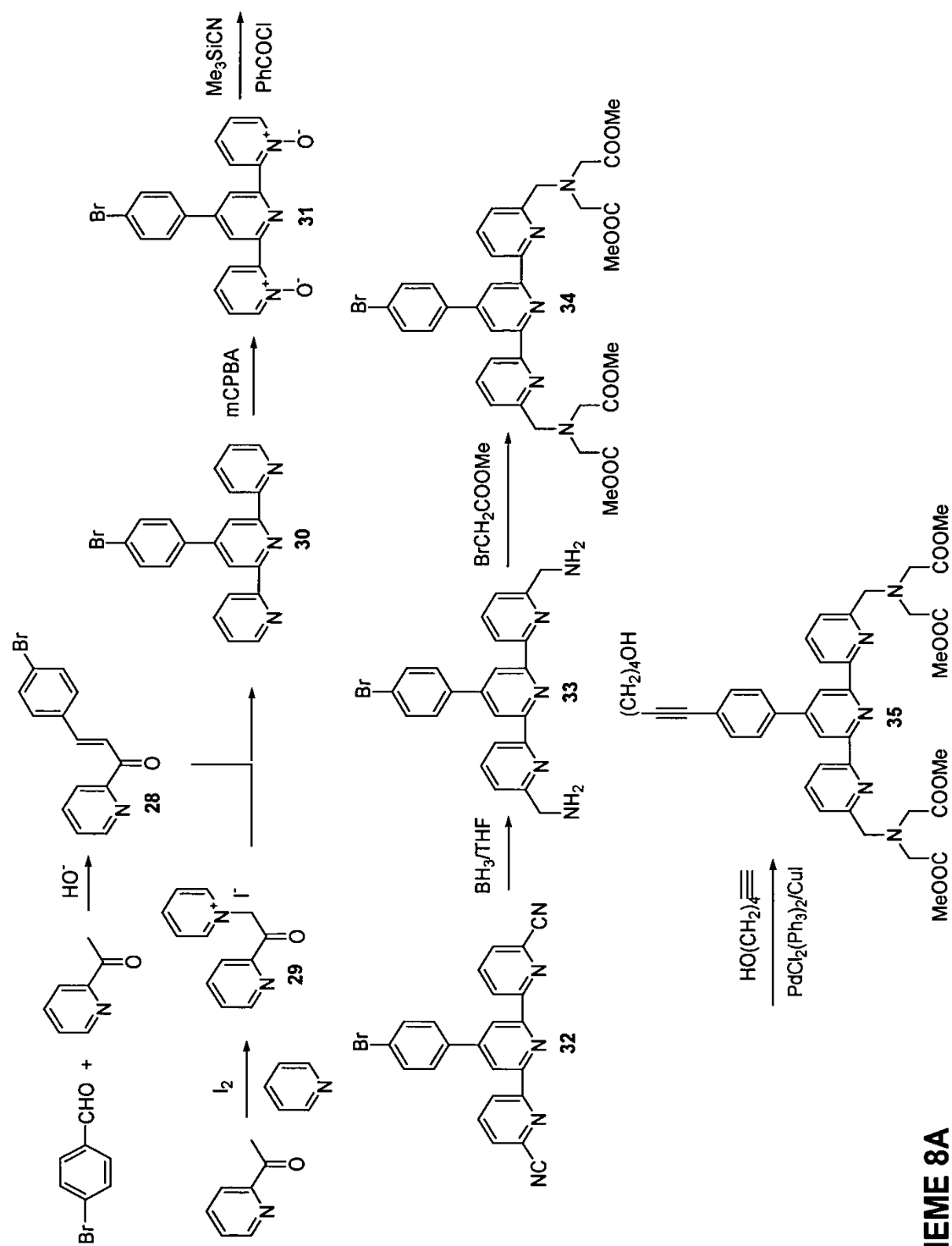
SCHEME 6B



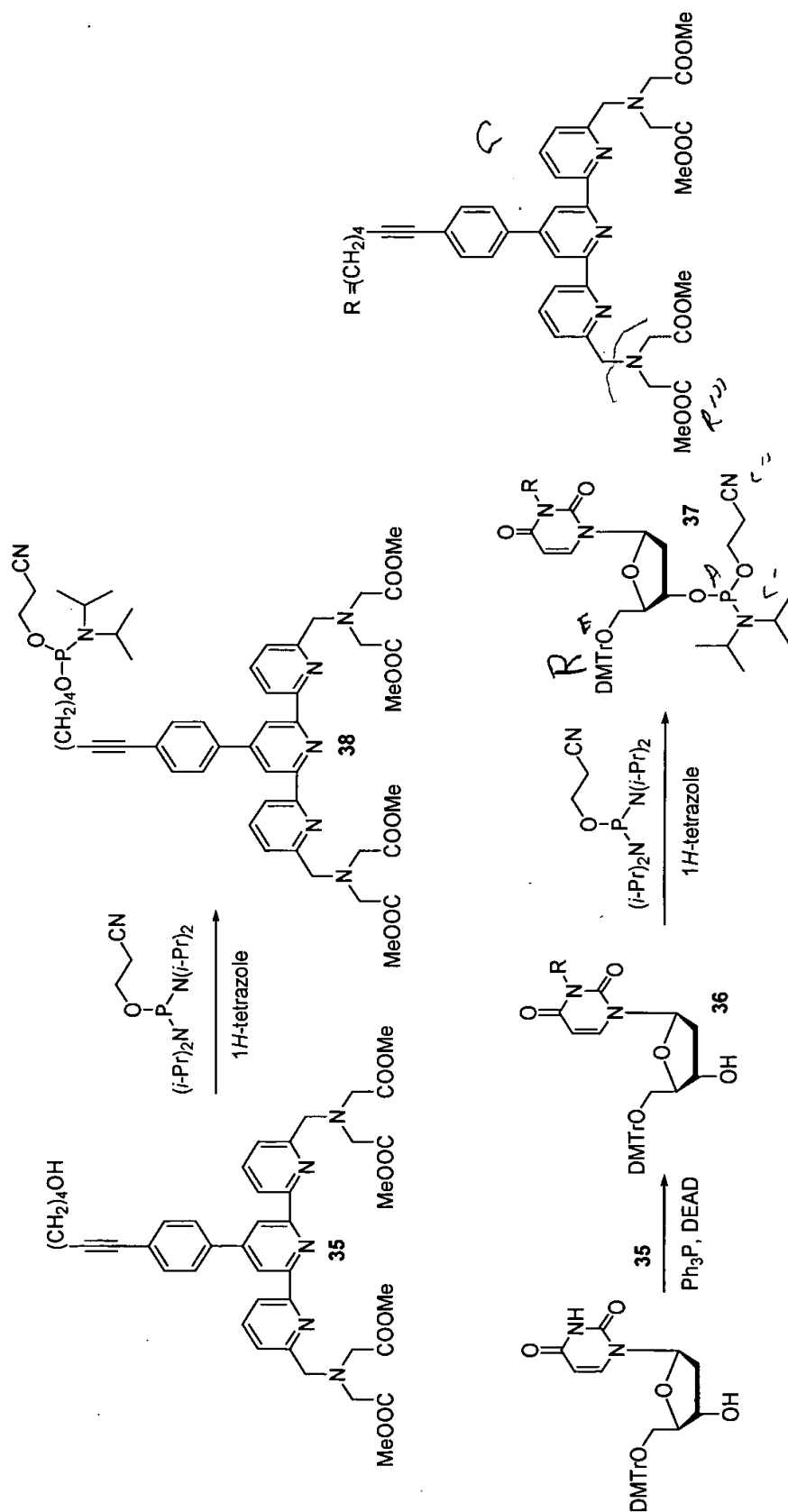
SCHEME 7A

## SCHEME 7B

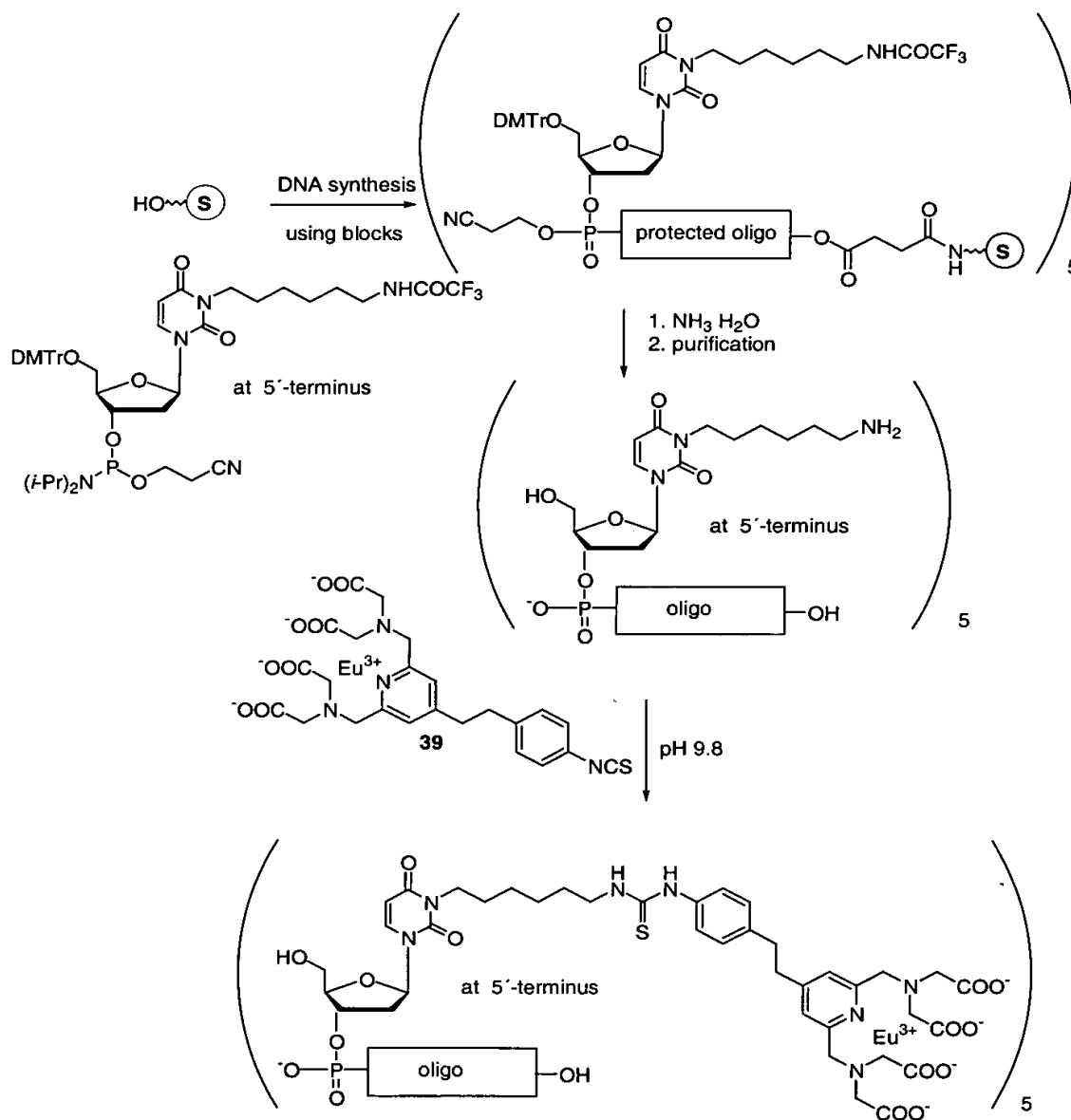




**SCHEME 8A**

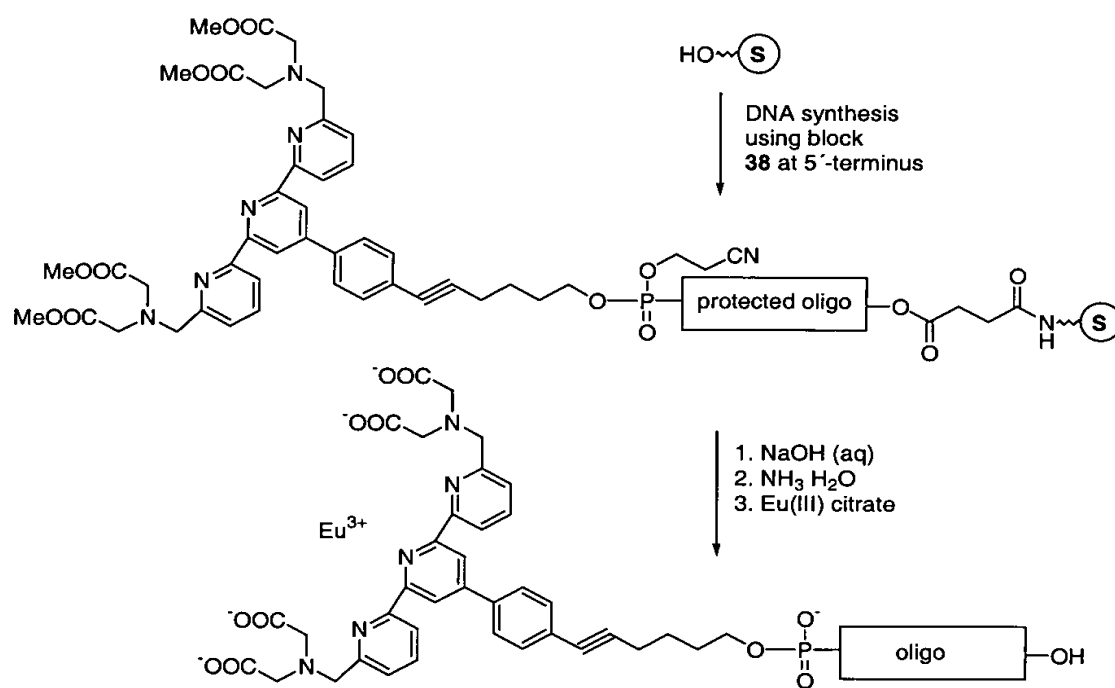


SCHEME 8B



SCHEME 9





SCHEME 11